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DRUG DISCOVERY TARGETING NON-AMYLOID PATHWAYS IN ALZHEIMER'S DISEASE

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Karolinska Institutet

Stockholm 2020

Published by Karolinska Institutet.
Printed by E-print AB, 2020

ISBN 978-91-7831-742-4

Cover image: Nerve growth factor (NGF) differentiated human fetal dorsal root ganglion (DRG) neurons stained with Hoechst nuclear stain (blue), and antibodies for β-tubulin (green) and phospho-ERK1/2 (red).

Drug discovery targeting non-amyloid pathways in Alzheimer's disease

THESIS FOR DOCTORAL DEGREE (Ph.D.)

by

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To my family

ABSTRACT

Alzheimer's disease (AD) is the most common form of dementia with almost 50 million people affected and unfortunately, no cure or treatment to halt the disease progression is available. In the hereditary forms of AD, an overproduction of amyloid β (Aβ) and the Aβ plaque formation are the basis for the amyloid hypothesis of AD, while several additional factors seem to influence the likelihood to develop sporadic forms of AD. Inflammation, mitochondrial dysfunction, neurotransmitter alterations, defective autophagy processes, the ApoE4 gene variant, oxidative stress and lack of neurotrophic support, are other established alterations associated with sporadic forms of AD.

The main aim of this thesis was to advance non-amyloidogenic drug discovery projects to improve the treatment options in AD.

The first target for drug discovery research in this thesis was the 15-lipoxygenase-1 (15-LO-1) enzyme, which oxidizes polyunsaturated fatty acids and forms both reactive oxygen intermediates and pro- as well as anti-inflammatory metabolites. The 15-LO-1 activity has been found upregulated in the brain of AD patients. Inhibitors of 15-lipoxygenase have therefore been suggested as potential therapeutic substances for AD. In Study I, we developed a high-throughput screening assay with the aim to identify 15-LO-1 inhibitors. An automated assay in 384-well format using fluorescent analysis amenable for high-throughput screening was established. Potent 15-LO-1 inhibitors were identified, which may be a useful tool to evaluate the inflammatory component of AD.

The second target for drug discovery approaches in this thesis was the altered neurotrophin signaling observed in AD. Basal forebrain cholinergic neuronal degeneration is one of the earliest signs of AD leading to cognitive impairment, described as the cholinergic hypothesis of AD. Associated with this impairment in the brains of patients with AD is the reduced nerve growth factor (NGF) signaling. The cognitive dysfunction seen in AD is also linked to the memory formation process in the hippocampus and the brain derived neurotrophic factor (BDNF) signaling. To modulate neurotrophin signaling, we investigated three NGF mutants in Study II and identified two small molecular compounds in Study III and IV that improve NGF and BDNF signaling.

In Study II we showed that the hereditary sensory and autonomic neuropathy type V (HSAN V)-like mutant (NGF-R100E), displayed more potent activation of the neurotrophin receptor TrkA and could promote cell survival and neurite outgrowth to a higher degree than wild-type NGF itself in human fetal dorsal root ganglion (DRG) neurons. In Study III we identified a class of steroid derivatives which potently enhanced both the TrkA and the neurotrophin receptor TrkB signaling. Compound AC-25793, a soluble cardenolide was investigated further, which attenuated cognitive dysfunction and decreased depressive-like symptoms *in vivo* in mice. In Study IV we discovered a registered drug that enhanced NGF and BDNF signaling, increased long-term potentiation in rat hippocampal slices, increased acetylcholine levels after microdialysis in rats, improved cognitive functions and reduced depression-like symptoms in both mice and rats. The results using this compound, named ACD855 by us, suggest a compound with promising potential for treating cognitive deficiency and depression in AD.

In summary, these findings have contributed to further knowledge in drug discovery projects aiming for a non-amyloid treatment in AD.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following papers, referred to in the text by their Roman numerals.

I. **Märta Dahlström***, Daniel Forsström*, Malin Johannesson, Yasmin Huque-Andersson, Marie Björk, Erik Silfverplatz, Andrei Sanin, Wesley Schaal, Benjamin Pelcman and Pontus Forsell. **Shared first authorship*.

   Development of a fluorescent intensity assay amenable for high-throughput screening for determining 15-lipoxygenase activity.

   Journal of Biomolecular Screening (2010) 15(6), 671-679

II. **Märta Dahlström**, Gunnar Nordvall, Erik Sundström, Elisabet Åkesson, Gunilla Tegerstedt, Maria Eriksdotter and Pontus Forsell.

   Identification of amino acid residues of nerve growth factor important for neurite outgrowth in human dorsal root ganglion neurons.

   The European Journal of Neuroscience (2019) 50(9), 3487-3501

III. **Märta Dahlström**, Gunnar Nordvall, Nather Madjid, Johan Sandin, Sven Ove Ögren, Maria Ankarcrona, Erik Sundström, Maria Eriksdotter and Pontus Forsell.

   The cardenolide AC-25793 enhances neurotrophin signaling and attenuates cognitive dysfunction in mice.

   Manuscript

IV. **Märta Dahlström**, Nather Madjid, Gunnar Nordvall, Erika Vazquez-Juarez, Maria Lindskog, Johan Lundkvist, Johan Sandin, Magnus Halldin, Bengt Winblad, Maria Eriksdotter and Pontus Forsell

   The effects of ACD855 on neurotrophin signaling, cognition and depression.

   Manuscript

CONTENTS

LIST OF ABBREVIATIONS

12-HETE	12-Hydroxy-(5,8,10,14)-eicosatetraenoic acid
13-HODE	13-Hydroxy-9,11-octadecadienoic acid
13-HPODE	13-Hydroperoxy-9,11-octadecadienoic acid
15-HETE	15-Hydroxy-(5,8,11,13)-eicosatetraenoic acid
15-HPETE	15-Hydroperoxy-(5,8,11,13)-eicosatetraenoic acid
5-LO	5-Lipoxygenase
15-LO-1	15-Lipoxygenase-1
%CV	Coefficient of variation
Aβ	Amyloid β
ACh	Acetylcholine
AChE	Acetylcholinesterase
AChEI	Acetylcholinesterase inhibitor
aCSF	Artificial cerebrospinal fluid
AD	Alzheimer's disease
ADAM10	Disintegrin and metalloproteinase domain-containing protein 10
AMPA	α-Amino-3-hydroxi-5-metyl-4-isoxazol-propansyra
ANOVA	Analysis of variance
Aph-1	Anterior pharynx defective-1
APP	Amyloid precursor protein
APOE	Apolipoprotein E gene
ApoE	Apolipoprotein E
BALB/c	Bagg albino inbred mouse strain
BDNF	Brain-derived neurotrophic factor
BFCN	Basal forebrain cholinergic neurons
BSA	Bovine serum albumin
C57BL/6J	C57 black 6 inbred mouse strain, originally from The Jackson Laboratory
CD	Candidate drug
CJD	Creutzfeldt-Jakob disease
CNS	Central nervous system
COX-1	Cyclooxygenase 1
COX-2	Cyclooxygenase 2

CSF	Cerebrospinal fluid
CT	Computed tomography
DFP	Diisopropyl fluorophosphate
DLB	Dementia with Lewy bodies
DMSO	Dimethyl sulfoxide
DPPP	Diphenyl-1-pyrenylphosphine
DRG	Dorsal root ganglion
EC_{50}	Half maximal effective concentrations
EFC	Enzyme fragment complementation
ELISA	Enzyme-linked immunosorbent assay
EOAD	Early-onset Alzheimer's disease
ERK1/2	Extracellular signal-regulated protein kinases 1 and 2
EX	Eoxin
FAD	Familial Alzheimer's disease
fEPSP	Field excitatory postsynaptic potentials
FSL	Flinders Sensitive Line
FST	Forced swim test
HD	Huntington's disease
HPLC	High-performance liquid chromatography
HSAN IV	Congenital insensitivity to pain with anhidrosis (CIPA)
HSAN V	Hereditary sensory and autonomic neuropathy type V
HTS	High-throughput screening
IC_{50}	Half maximal inhibitory concentration
IL-1β	Interleukin 1 β
L1236	Hodgkin lymphoma cell line
LTP	Long-term potentiation
LX	Lipoxin
MCI	Mild cognitive impairment
MMSE	Mini-mental state examination
MRI	Magnetic resonance imaging
NGF	Nerve growth factor
NGF	Nerve growth factor gene
NMDA	N-metyl-D-aspartat
NMRI	Albino outbred mouse strain originally from Naval Medical Research Institute

NT-3	Neurotrophin-3
NT-4/5	Neurotrophin-4/5
P-tau	Hyperphosphorylated tau
PBS	Phosphate-buffered saline
PC12	Rat adrenal gland pheochromocytoma cell line
PCN	Primary cortical neurons
PD	Parkinson’s disease
PDL	Poly-D-lysine
pERK1/2	Phosphorylated ERK1/2
PET	Positron-emission tomography
PSEN1	Presenilin protein 1
PSEN2	Presenilin protein 2
PVDF	Polyvinylidene difluoride
RP-HPLC	Reverse-phase HPLC
RPMI	Roswell Park Memorial Institute
S/B	Signal-to-background
SAR	Structure-activity-relationship
SC	Schaffer collaterals
SD	Standard deviation
SEM	Standard error of the mean
SORL1	Sortilin related receptor 1
SPM	Specialized pro-resolving mediator
TBS	Tris-buffered saline
TNFα	Tumor necrosis factor α
TREM2	Triggering receptor expressed on myeloid cells 2
TrkA	Tropomyosin related kinase A
TrkB	Tropomyosin related kinase B
TrkC	Tropomyosin related kinase C
U2OS	Human bone osteosarcoma cell line
wt	Wild-type
Z’	Z-prime factor

1 INTRODUCTION

1.1 NEURODEGENERATION

Neurodegeneration in the central nervous system (CNS) is part of the normal aging process but found accelerated and specified to certain brain areas in neurodegenerative disorders, with selective loss of neurons and specific loss of functions. Mutations in a broad variety of both related and unrelated genes are linked to hereditary forms of neurodegenerative diseases. Although a majority of the observed cases are sporadic, there are also many examples of familial neurodegenerative diseases. One hallmark of certain neurodegenerative diseases is the accumulation and deposition of misfolded proteins that cause toxic aggregates. In Alzheimer's disease (AD) an accumulation and deposition of both amyloid β (Aβ) and hyperphosphorylated tau (p-tau) protein occur. Parkinson's disease (PD) is recognized by misfolded alpha-synuclein, Creutzfeldt-Jakob disease (CJD) by infectious prion proteins, dementia with Lewy bodies (DLB) by misfolded inclusions identified with ubiquitin antibodies and in Huntington's disease (HD) mutated and misfolded huntingtin protein form toxic aggregates. Despite the differences in symptomatology and underlying molecular pathways, several neurodegenerative diseases share common features, unfortunately also that they so far are incurable, with only partial symptomatic treatments available.

1.2 ALZHEIMER'S DISEASE

Alois Alzheimer examined 1901 a middle-aged woman with specific memory deficits and progressive loss of cognitive abilities, including poor spatial orientation and suspicion. Following her death five years later, an autopsy of the brain showed distinct morphological alterations, now known to be extracellular Aβ plaques and intracellular neurofibrillary p-tau tangles. This form of dementia was subsequently given the name Alzheimer's disease (Alzheimer, 1907; Alzheimer et al., 1995).

AD is the most common form of dementia with almost 50 million people affected worldwide (alzheimers.net), corresponding to about 50-75% of all dementia cases (Lane et al., 2018). Dementia, including AD, is a major cause of disabilities and the overall burden from AD is considerable, with encountered patients and family members, and immense social costs. With increasing life expectancy, the number of people with AD is expected to triple by 2050 (Lane et al., 2018). About 175,000 people in Sweden are today living with a dementia diagnosis, where 68% of people identified with dementia in 2018 were diagnosed with AD or mixed AD (*SveDem, Yearly report,* 2018). The financial burden for the Swedish society connected to

dementia corresponds to the costs of cancer, cardiovascular disease and stroke together ("www.alzheimersverige.se," 2020).

1.2.1 Stages

There is increasing evidence demonstrating that AD has a long preclinical phase (10-20 years) with neuronal loss and changes in biomarkers (Masters et al., 2015). Decreased cerebrospinal fluid (CSF) $A\beta_{1-42}/A\beta_{1-40}$ ratio is linked both to high Aβ deposition in the brain, with increased risk to develop AD, as well as with the clinical phase of AD. Levels of total tau and phosphorylated tau is increased in CSF and in the brain in AD. Both Aβ and tau can be visualized using positron-emission tomography (PET) imaging (Villemagne et al., 2018).

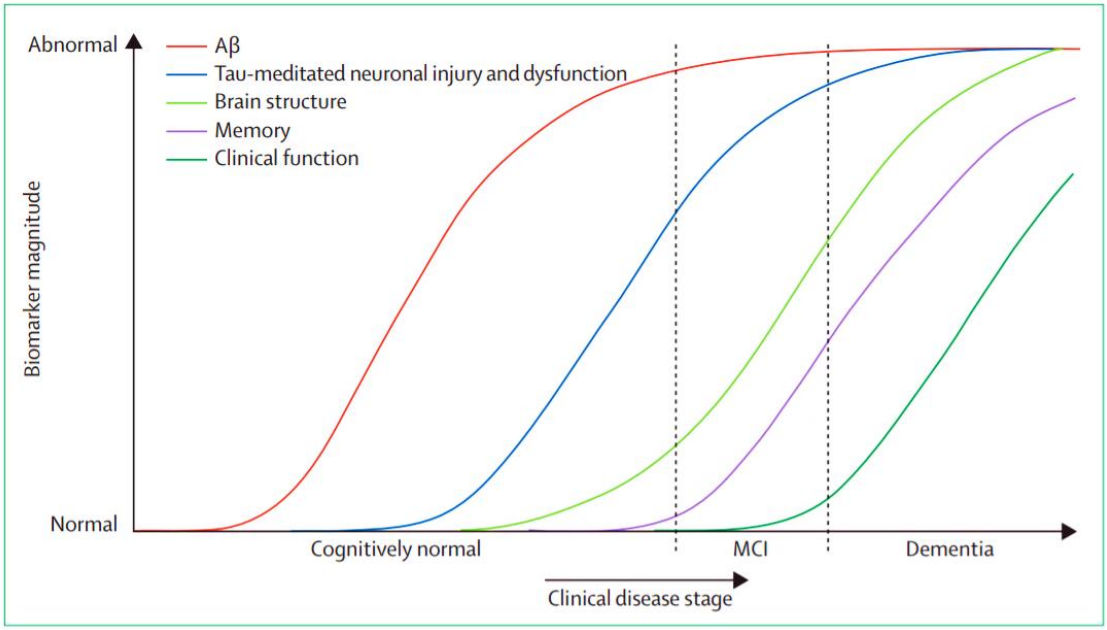

Figure 1. Biomarkers and pathophysiology of AD. A proposed hypothetical model of biomarkers related to the clinical stages in AD. Aβ and tau is measured in CSF or assessed with PET. Adapted with permission (Jack et al., 2010).

The preclinical stage of AD eventually develops to mild cognitive impairment (MCI) which usually involves subtle decline in episodic memory, problems with independence, difficulties planning and completing a task, without considerably interfere with daily life activities, but where the decline is greater than in the expected cognitive weakening of normal aging (Winblad et al., 2004). A person with MCI has an increased risk of developing AD, but MCI can also reverse to a normal stage or advance to other dementias (Michaud et al., 2017).

The clinical symptoms associated with AD are gradual loss of memory, further problems with planning and problem-solving, poor judgment, changes in personality, confusion,

aggressiveness, language and orientation problems, social withdrawal, depression, problems with daily activities, suspicion and illusions. The symptoms may vary from person to person, while the characteristic of dementia is the progressive decline over time.

1.2.2 Diagnosis

Diagnosing AD normally starts with an exclusion of other disorders. In Sweden the diagnostic work-up for dementia is recommended in the National guidelines published by the Swedish Board of Health and Welfare. When suspecting memory decline, a basal dementia work-up should be performed, including history from patient and caregiver, a Mini-mental state examination (MMSE), clock test, functional assessment and a computed tomography (CT) scan of the brain (*Swedish Board of health and welfare. National guidelines on dementia care, 2010, revised*, 2017). MMSE is a questionnaire that test memory, language, orientation, attention, ability to plan and visual abilities, but does not test the mood swings often related to AD (Folstein et al., 1975). The maximum score is 30 with an estimated decline in MMSE score of 3-4 points per year in untreated and 2.5 points per year in AD patients treated with a cholinesterase inhibitor (Han et al., 2000; Lopez et al., 2002). Dementia has been staged based roughly on MMSE scores, i.e. suggested mild dementia with an MMSE score of ≥ 20, suggested moderate dementia with an MMSE score of 10-19 and suggested severe dementia with an MMSE score of <10. A draw back with the absolute number of MMSE score, is that it is affected by the person's education level and the ability to master the language. A more extensive investigation is performed at memory clinics including a biomarker investigation of CSF after lumbar puncture, magnetic resonance imaging (MRI) and thorough cognitive testing. The average survival length after a clinical diagnosis of AD is 4-8 years in people over 65 years of age, but individuals may live up to 20 years after diagnosis (Alzheimer's Association, 2016; Garcia-Ptacek et al., 2014). Bronchopneumonia has been found to be the most common cause of death in AD patients, followed by ischemic heart disease (Brunnström and Englund, 2009), while data on causes of death on individuals with dementia from the Swedish Dementia Registry (SveDem) showed that the most common cause of death was ischemic heart disease (Garcia-Ptacek et al., 2016).

1.2.3 Genetics and risk factors

Age is the strongest risk factor for developing sporadic AD and old women are at special risk (Fratiglioni et al., 1997). At 65 years of age, about 1-3% of the population is presumed to have AD and after 65 years of age, the incidence of AD increases exponentially (Masters et al., 2015). Less than 1% of AD cases are hereditary forms (Masters et al., 2015) and the majority

of the mutations causing AD are situated in the amyloid precursor protein (APP), or in the presenilin protein 1 or 2 (PSEN1 or PSEN2) genes. These mutations are autosomal dominant and cause early-onset AD (EOAD), also known as familial Alzheimer's disease (FAD) (Lanoiselée et al., 2017). These mutations predominantly cause an overproduction of Aβ, especially increased production of $A\beta_{1-42}$ (Masters et al., 2015). Another genetic risk factor for AD is trisomy 21 (Down syndrome). Individuals with Down syndrome has a triplication of the APP gene, located on chromosome 21q21. The increased production of APP and Aβ in these individuals lead to an increased risk to develop EOAD (Hartley et al., 2015). FAD exhibits a penetrance that is close to 100%, with a relatively predictable age of onset from family to family, and is usually more aggressive than late onset AD (Mendez, 2017).

The most common genetic risk factor for sporadic or late onset AD is the *APOE* ε4 gene variant of the apolipoprotein E (ApoE), a protein with three isoforms, *APOE* ε2, *APOE* ε3 and *APOE* ε4. The risk to develop AD is predicted to be three times higher for heterozygous *APOE* ε4 carriers and 15 times higher for homozygous *APOE* ε4 carriers compared with the *APOE* ε3 variant, which is the most common genetic variant, while *APOE* ε2 reduces the risk to develop AD (Van Cauwenberghe et al., 2016). The main function of the ApoE protein in the brain is to transport lipids and cholesterol to the neurons. The ApoE protein also binds to Aβ and facilitates Aβ clearance from the brain, a process where the ApoE4 protein is assumed to be less effective.

There are at least 20 other genes linked to AD, for example the disintegrin and metallo-proteinase domain-containing protein 10 (ADAM10), triggering receptor expressed on myeloid cells 2 (TREM2) and sortilin related receptor 1 (SORL1) (Hardy et al., 2014; Van Cauwenberghe et al., 2016). A number of lifestyle factors have also been identified to affect the risk of developing late onset AD. Positive influences from a balanced died, higher education, mental challenges, physical exercise and social activity have been shown. On the other hand increased risks for sporadic AD have been shown to be linked to stress, sleep disturbances, diabetes, cardiovascular diseases, high-fat or high-sugar diets, alcohol abuse, smoking and high blood pressure (Fratiglioni et al., 1993; Winblad et al., 2016).

1.3 ALZHEIMER'S DISEASE PATHOLOGY

Early events in the pathogenesis of AD are disturbed cholinergic function and synaptic dysfunction, both of which are highly correlated with cognitive dysfunction. As the disease progresses, neuroinflammation, neuronal death, and loss of synaptic connections with subsequent shrinkage of the brain occur (Winblad et al., 2016). The AD brain pathology is

characterized by atrophy, causing reduced brain volume, enlarged ventricles and hippocampal atrophy, with widespread neuronal death and with extracellular Aβ plaques and intracellular neurofibrillary tangles (Deture and Dickson, 2019). According to the amyloid hypothesis, accumulation and deposition of Aβ peptides is the driving pathology in AD (Selkoe and Hardy, 2016).

1.3.1 Amyloid β

APP is widely distributed in the human body and is found abundant in the synapses of neurons. APP is sequentially cleaved first by α-secretase or β-secretase and then further cleaved by γ-secretase, producing the P3 fragment after α-secretase cleavage or the Aβ peptide after β-secretase cleavage. The γ-secretase is a protein with four main subunits, PSEN1, PSEN2, nicastrin and anterior pharynx defective-1 (Aph-1) (Sherrington et al., 1995). The α-secretase cleavage is called the non-amyloidogenic pathway, since the P3 fragment is not prone to aggregate, and is also the main route under normal conditions. The β-secretase pathway, the amyloidogenic pathway, results in increased production of Aβ peptides, and is believed to have a fundamental role in the AD pathogenesis (Zhou et al., 2011). The Aβ fragments may be of variable length, $A\beta_{1-40}$ being the most common and $A\beta_{1-42}$ being the most toxic and with the highest susceptibility to aggregate. (Selkoe, 2002).

1.3.2 Tau

P-tau protein that form intracellular neurofibrillary tangles are, beside Aβ plaques, a key pathological feature of AD. Tau phosphorylation is suggested to be secondary to Aβ accumulation in AD, since mutations related to FAD give rise to increased Aβ production and increased p-tau. Tau proteins are highly soluble microtubule-associated proteins that stabilize the microtubules in neurons and are vital for neuronal functions and axonal transport (Weingarten et al., 1975). The most common post-translational regulation of tau is via phosphorylation of its serine or threonine residues (Martin et al., 2011), regulated by kinases, an event that causes tau to detach from the microtubules. P-tau protein form insoluble intracellular neurofibrillary tangles in the form of paired helical filaments found in AD. The tau pathology observed in AD is probably a combination of loss of normal tau function and a gain of toxic effect of the neurofibrillary tangles (Ballatore et al., 2007; Iqbal et al., 2014).

1.3.3 Neuronal degeneration and the basal forebrain cholinergic system

The synapses are the terminals were neurons communicate with each other, the utmost delicate part of the neuronal system. Synapses constantly modulate and adapt to the signaling pattern

in the brain. Due to their high ability to adapt, synapses are also highly vulnerable to toxic or environmental changes. Synaptic dysfunction and synaptic protein changes are early markers of AD pathogenesis (Spires-Jones and Hyman, 2014).

Synaptic loss and neuronal degeneration, especially in the basal forebrain cholinergic neurons (BFCN) and their projections to hippocampus and cerebral cortex, are linked to early signs of cognitive decline and AD. This is described as "The cholinergic hypothesis of AD" (Bartus et al., 1982). Memory, learning and other cognitive functions transmitted via cholinergic neurons are dependent on the neurotransmitter acetylcholine (ACh). The levels of ACh are decreased in AD which is linked to degeneration of cholinergic neurons, reduced synthesis of ACh by choline acetyltransferase (ChAT), and to decreased neurotrophic support from nerve growth factor (NGF) signaling (Rinne et al., 1988; Schliebs and Arendt, 2011). The acetylcholinesterase (AChE) is responsible for the break-down of ACh in the synaptic cleft, a mechanism that reduce prolonged or excessive firing of the post-synaptic neuron via the muscarinic and nicotinic ACh receptors. To compensate for the decreased support from ACh signaling in AD, acetylcholinesterase inhibitors (AChEI) were developed as treatments in mild to moderate AD and are hitherto the only drugs in addition to the N-metyl-D-aspartat (NMDA)-receptor antagonist memantine (see below) for the symptomatic treatment of AD (Knight et al., 2018).

In behavioral studies, the drug scopolamine is used to block the muscarinic ACh receptors to mimic the cognitive dysfunction in AD (Bartus, 2000; Bymaster et al., 1993). Scopolamine blocks both postsynaptic muscarinic receptors (mainly M1 and M3) and presynaptic muscarinic receptors (mainly M2 and M4). Scopolamine binding to postsynaptic muscarinic receptors prevents ACh signaling, which impairs learning and memory. Scopolamine binding to presynaptic muscarinic receptors reduces the negative feed-back of ACh release, causing a continuous release of ACh from the presynaptic neuron and an increased ACh signaling via nicotinic receptors, leading to over-activation of the nicotinic receptor and additional reduced cognitive functions (Newman and Gold, 2016).

Another neurotransmitter with essential cognitive functions is glutamate. In the memory formation process, presynaptic neuronal stimulation releases glutamate, which signals via the α-amino-3-hydroxi-5-metyl-4-isoxazol-propansyra (AMPA) receptor. The glutamate activation of AMPA receptors causes an inflow of Na^+ ions into the post-synaptic cell, which recruits more AMPA receptors. The increased intracellular Na^+ concentrations and the high glutamate signaling activate post-synaptic glutamate/NMDA signaling, leading to an inflow of Ca^{2+} into the post-synaptic cell. The inflow of Ca^{2+} propagates the firing in the post-synaptic

neuron and induce effectors for synaptic plasticity, learning and memory formation, for example brain-derived neurotrophic factor (BDNF).

Glutamate signaling via synaptic NMDA receptors has been shown to promote synaptic plasticity and neuronal survival, while excessive glutamate signaling via extrasynaptic NMDA receptors has been shown to induce cell death (Olivares et al., 2012). In animal models, the drug MK-801 is used to block NMDA receptors causing a memory deficiency with reduced glutamate signaling and impaired long-term potentiation (LTP), mimicking the disturbed glutamate signaling found in AD (Foster and Fagg, 1987; Huettner and Bean, 1988).

1.3.4 Inflammation

Inflammation with chronic immune reactions is found in diseased areas of the AD brain and therefore neuroinflammation has evolved as a third central pathology in AD (Kinney et al., 2018), where an upregulation of cytokines mediates the pro-inflammatory state in the brain, and the main inflammatory mediators are tumor necrosis factor α (TNFα), interleukin 1 β (IL-1β) and IL-6 (Su et al., 2016). Arachidonic acid, a polyunsaturated fatty acid, and its down-stream metabolites are other key players involved in the immune response. Several well-known pathways and therapeutic targets are found within the arachidonic acid cascade, which have implications for the neuronal inflammatory process (AlFadly et al., 2019). Another significant contributing factor to the immune response is the microglial cells, which protect the brain from toxic proteins by clearing it from Aβ plaques, while in the diseased stage, large numbers of dysfunctional activated microglia are found within the lesions of AD (Heela and Heneka, 2017; Streit et al., 2020). The final stage of inflammation is the resolution stage, which involve specialized pro-resolving mediators (SPMs) for example lipoxins (LXs), which is found dysfunctional and may contribute to the chronic inflammations found in AD (Wang et al., 2015).

1.4 15-LIPOXYGENASE-1

Lipoxygenases are iron-containing oxidizing enzymes in the lipid metabolism, using the catalytic ion to incorporate molecular oxygen into unsaturated fatty acid. One of the main substrates for lipoxygenases is arachidonic acid. The lipoxygenases are named after where on arachidonic acid they introduce the molecular oxygen (Funk et al., 2002). Arachidonic acid metabolizing enzymes like cyclooxygenase 1 (COX-1), cyclooxygenase 2 (COX-2) and 5-lipoxygenase (5-LO) are well known for their production of pro-inflammatory metabolites, like the prostaglandins and the leukotrienes (Bergström and Samuelsson, 1962; Samuelsson, 1983). There are three major lipoxygenases found in humans, 5-LO, 12-LO and 15-LO with

two different forms of 15-LO, named 15-LO-1 or 15-LO-2. The human 15-LO-1 enzyme corresponds to animal 12/15-LO, formerly called leukocyte type 12-LO. Mice, rats and pigs express the 12/15-LO (Takahashi et al., 1993). Rabbit reticulocytes express 15-LO-1, which is the only animal species known to express 15-LO-1. The human 15-LO-1 converts arachidonic acid to 15-hydroxy-(5,8,11,13)-eicosatetraenoic acid (15-HETE) and 12-hydroxy-(5,8,10,14)-eicosatetraenoic acid (12-HETE) in a 9:1 ratio, while the animal 12/15-LO converts arachidonic acid mainly to 12-HETE (Kuhn et al., 2002). 15-LO-2 has little in common with 15-LO-1, and is found in skin, cornea, lung and prostate (Brash et al., 1997).

The human 15-LO-1 is selectively expressed in tissues like reticulocytes, eosinophils, airway epithelial cells and in the CNS (Joshi et al., 2015; Kühn and O'Donnell, 2006). The roles of 15-lipoxygenase type 1 (15-LO-1) in humans are not as well investigated, as the role of 12/15-LO in animals. Nevertheless, 15-LO-1 has been implicated in cytokine release from airway epithelial cells, asthma, cardiac inflammation, atherosclerosis and AD (Feltenmark et al., 2008; Praticò et al., 2004). Both pro- and anti-inflammatory actions are observed for 15-LO-1 (Kühn and O'Donnell, 2006). The arachidonic acid metabolic pathway via 15-lipoxygenase-1 is shown in Figure 2.

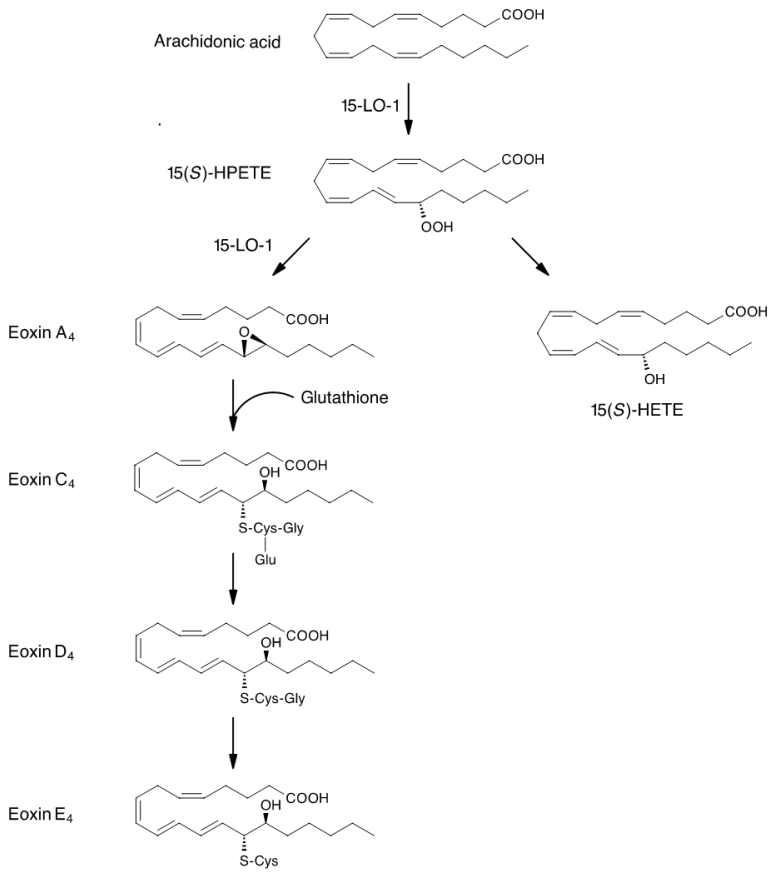

Figure 2. The arachidonic acid metabolic pathway via 15-lipoxygenase-1, modified from Feltenmark *et al.* (Feltenmark et al., 2008). 15-LO-1 metabolize arachidonic acid by catalyzing the incorporation of molecular oxygen into arachidonic acid at carbon 15, producing 15-HPETE and by hydrolyzing 15-HPETE to EXA_4.

The arachidonic acid derived cysteinyl containing 15-LO-1 metabolites, the eoxins (EX) were first discovered in human eosinophils (Feltenmark et al., 2008). The EXs have been shown to display pro-inflammatory actions in mast cells, eosinophils and lung epithelial cells (Feltenmark et al., 2008; Liu et al., 2009). The anti-inflammatory actions of 15-LO-1 is likely mediated via the arachidonic acid derived metabolites LXs (Sobrado et al., 2009). LXs are associated with suppression and resolution of inflammation. The chronic inflammation found in AD is linked to impaired resolution, where the pro-resolving mediator LXA_4 is down-regulated (Wang et al., 2015).

Emerging evidence has demonstrated the involvement of 15-LO-1 in AD (Di Meco et al., 2017; Joshi et al., 2015). 15-LO-1 has been shown to be significantly upregulated in the brains of persons with AD or MCI (Praticò et al., 2004; Yao et al., 2005). 15-LO-1 has the ability to oxygenate both free fatty acids and fatty acids bound in membranes, which may lead to membrane damage and thereby contribute to inflammatory processes and neurodegeneration. The 15-LO-1 metabolites 12-HETE and 15-HETE are also important messenger molecules in the communication at the synapse, which may cause memory deficits with increased 15-LO-1 expression (Piomelli et al., 1987). Oxidative stress occurs when there is an excess of free radicals, and increasing evidence suggest that oxidative stress is an early marker of the AD pathogenesis. The hydroperoxides, 15-hydroperoxy-(5,8,11,13)-eicosatetraenoic acid (15-HPETE) and 13-hydroperoxy-9,11-octadecadienoic acid (13-HPODE), formed when 15-LO-1 oxidize arachidonic acid or linoleic acid, are potent mediators of oxidative stress and the subsequent neurodegeneration (Praticò et al., 2004). Furthermore, it has been shown in transgenic mice models of AD that overexpression of 12/15-LO augment the amyloid pathology, increase soluble tau levels and negatively affected memory functions, which could be ameliorated with a specific 12/15-LO inhibitor (Chu et al., 2015, 2012; Yang et al., 2010).

Several attempts to target 15-LO-1 in the treatment of AD have been performed. AlFadley *et al*. performed a multi-target approach by targeting AChE, COX-2 and 15-LO-1 simultaneously, in line with the described deficient ACh signaling and neuroinflammation found in AD (AlFadly et al., 2019). A COX-2 and 15-LO-1 inhibitor could potentially reduce the inflammatory response without the gastrointestinal side effects related to NSAID treatment.

1.5 NEUROTROPHINS

NGF, BDNF, neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5) belong to the neurotrophin family and signal via their respective high affinity tropomyosin related kinase (Trk) receptors and the low affinity p75 neurotrophin receptor (P75NTR, also known as p75) (Barde et al., 1982; Ernfors et al., 1990; Hallböök et al., 1991; Levi-Montalcini, 1952). For neurotrophin receptor specificity, see figure 3.

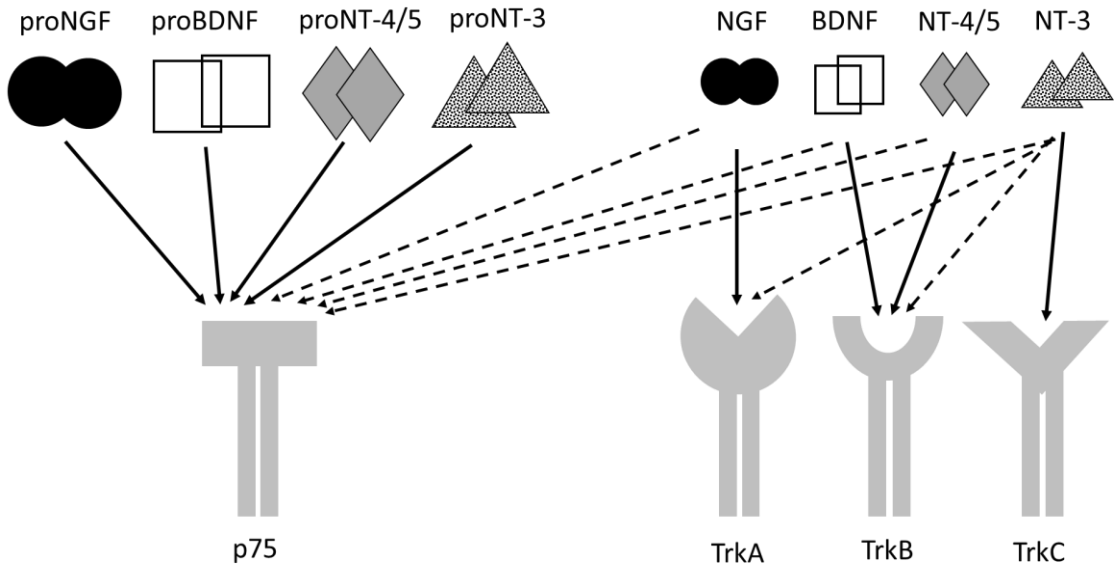

Figure 3. Neurotrophin signaling, where high affinity ligand binding is represented with a solid line and low affinity ligand binding with a dashed line. Pro-neurotrophins signal mainly via p75 causing apoptosis, while the mature neurotrophins signal via TrkA, TrkB and TrkC, according to figure, leading to cell survival events.

Neurotrophin binding to Trk receptors can initiate down-stream signaling that may lead to cell survival, proliferation, differentiation, and synaptic plasticity, among other functions, while neurotrophin signaling via p75 mainly promote an apoptotic signaling pathway.

The Trk receptors are cell surface receptors which are believed to dimerize upon neurotrophin binding, followed by an autophosphorylation of the receptor and then internalization of the neurotrophin-receptor complex (Franco et al., 2020), or shown by others to initially signal as a monomer and dimerize after internalization (Zahavi et al., 2018). The internalization of NGF/TrkA or BDNF/TrkB involves the formation of endosomes which mediate further signaling and retrograde transport of the complex, or may undergo recycling/degrading in the axon terminals (Marlin and Li, 2015; Yamashita and Kuruvilla, 2016). All the neurotrophins are expressed in a pro-form (immature neurotrophins), which are later cleaved to the mature proteins. Both the pro-forms and the mature forms of the neurotrophins are mainly found as

homodimers, where the molecular weight of the pro-form monomer is about 32 kDa and the mature monomer is about 13.5 kDa (Fahnestock et al., 2001). The high affinity receptor of the pro-forms of the neurotrophins is the p75 receptor (Kaplan and Miller, 2000). For a suggested schematic neurotrophin down-stream signaling pathway, see figure 4.

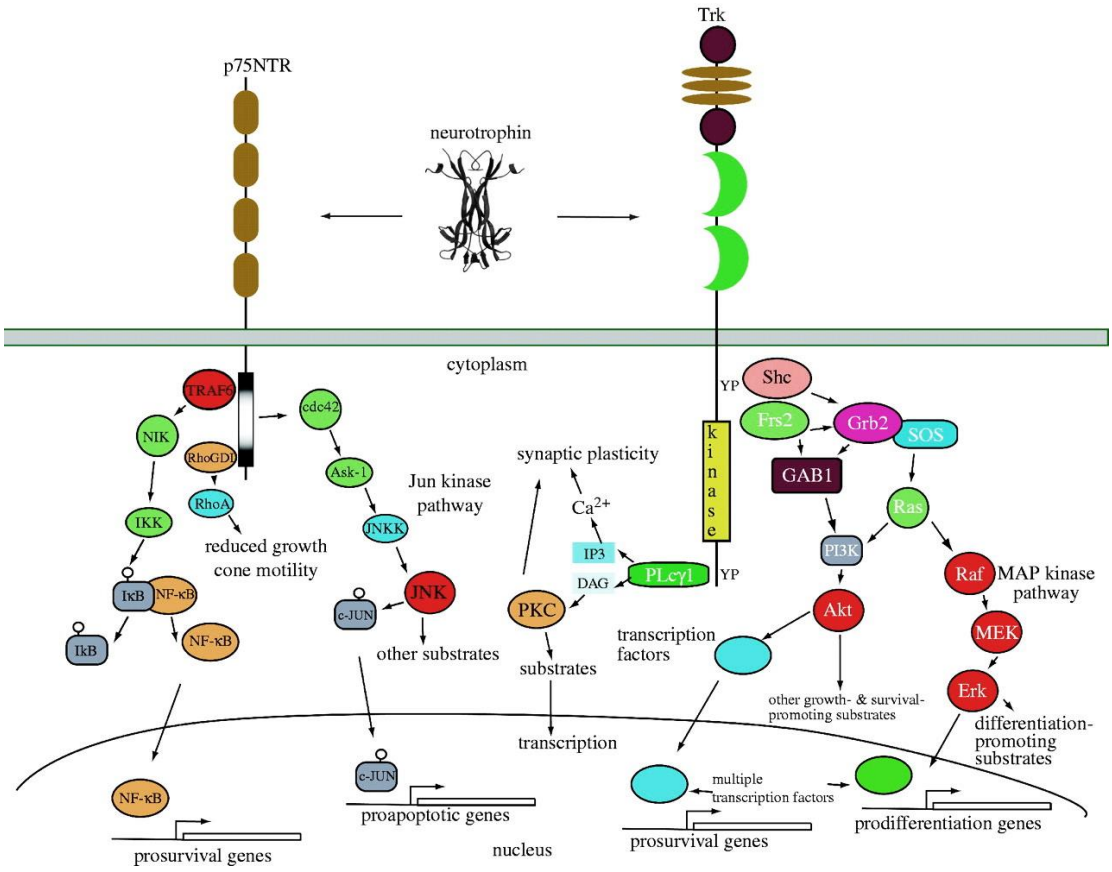

Figure 4. Schematic representation of neurotrophin down-stream signaling pathways via p75 and Trk receptors, and subsequent intracellular events. Adapted with permission (Reichardt, 2006).

NGF is essential for nerve development prenatally and in the adult, BFCN maintain their reliance on NGF for survival and function (Hefti, 1986). In the healthy condition, the balance between pro-NGF and mature NGF proteins and their signaling via TrkA and p75 maintain the right level of support to the neurons for survival, differentiation, proliferation and apoptosis (Fahnestock and Shekari, 2019). The early and selective loss of BFCN in AD (Davies and Maloney, 1976) is linked to decreased support from NGF (Salehi et al., 2004; Seiler and Schwab, 1984). Several events related to NGF affect the cholinergic functionality during the AD pathogenesis such as altered NGF maturation, skewed TrkA/p75 receptor ratio, inefficient axonal transport and signaling, Aβ induced modulation of NGF receptors and deficient ACh signaling (Mitra et al., 2019). The proNGF/NGF ratio is increased in AD patients (Fahnestock

et al., 2001), which leads to reduced retrograde transport of mature NGF from hippocampus and cortex to BFCN, and increased signaling via the p75 receptor causing increased apoptosis (Ito and Enomoto, 2016; Mufson et al., 2019). Lower levels of TrkA receptors in the basal forebrain and in cortex of AD patients further decrease the reduced NGF/TrkA signaling in the cholinergic neurons (Mufson et al., 1997). Furthermore, it has been shown in transgenic AD11 mice, which express an anti-NGF antibody, that deprivation of mature NGF induces inflammation and evokes AD-like neurodegeneration with Aβ plaques and neurofibrillary tangles, suggested to be linked to the evoked proNGF/NGF imbalance (Capsoni et al., 2011a, 2000). NGF is also linked to the Aβ pathology found in AD patients. NGF has been shown to directly reduce Aβ generation, and Aβ may prevent NGF maturation, which indicate how reduced NGF levels in AD patients might worsen the Aβ linked AD pathology (Bruno et al., 2009; Mitra et al., 2019). The NGF-dependent vulnerability of BFCN degeneration in AD is suggested to augment the disease, which is proposed to be linked to the skewed proNGF/NGF ratio (Capsoni and Cattaneo, 2006; Cuello et al., 2019).

High expression of BDNF and TrkB is mainly found in the human brain where it displays a significant impact on synapse plasticity and neurogenesis (Miranda et al., 2019). BDNF is defined as one of the most important factors for memory formation (Bekinschtein et al., 2014). Polymorphism in the proBDNF gene, the BDNF-Val66Met genotype, has been shown to cause lower levels of BDNF and is linked to significant increase in cognitive decline and hippocampal atrophy in both sporadic AD and FAD (Lee et al., 2013; Lim et al., 2018, 2016, 2013). Decreased levels of BDNF is also linked to depression, attention deficits, less stress tolerance, poorer weight control, insulin resistance and ADHD (Chan et al., 2019; Luo et al., 2020; Malhi and Mann, 2018; Motamedi et al., 2017; Notaras and van den Buuse, 2020), and BDNF levels are found increased after conventional anti-depressant treatment (Björkholm and Monteggia, 2016; Notaras and van den Buuse, 2020).

Both dementia and depression is common in the older population, and the comorbidity between the two disorders is extensive (Enache et al., 2016). The underlying reasons for the relationship between depression and neurodegenerative disorders, such as AD, is not fully understood, but there has been found similar neurodegenerative features in the brain of persons with dementia and depression (Bennett and Thomas, 2014), with strong correlations to inflammation and decreased levels of BDNF (Chi et al., 2014).

Intensive exercise has been shown to increase plasma BDNF levels (Jeon and Ha, 2017; Marquez et al., 2015; Szuhany et al., 2015; Wang and Holsinger, 2018). It is not fully elucidated from where the exercise induced BDNF is derived. Both peripheral and central BDNF levels

have been shown to be increased after physical exercise in humans (Rasmussen et al., 2009), while animal studies have shown that exercised induced BDNF expression in the brain possibly is mediated via metabolic changes in the liver (Sleiman et al., 2016).

The involvement of BDNF in hippocampal LTP is now well established (Lu et al., 2014; Woo and Lu, 2009). A long-lasting memory formation is dependent on BDNF expression (Navakkode and Korte, 2012), where lower serum levels of BDNF observed in AD patients (Ng et al., 2019) correlate with induced hippocampal atrophy and cognitive decline (Lim et al., 2013). Besides the memory formation processes dependent on BDNF, the toxic effects of Aβ in the AD brain are more pronounced with lower expression of BDNF, as seen with the BDNF-Val66Met polymorphism (Lim et al., 2016) and the neurotoxicity seen with excessive glutamate signaling via NMDA receptors is reduced if BDNF is present (Lau et al., 2015).

When NGF binds to TrkA or when BDNF binds to TrkB, a down-stream signaling cascade that can activate ERK1/2 (extracellular signal-regulated protein kinases 1 and 2), also known as mitogen-activated protein kinases (p42/p44 MAPK), which is part of the Ras-Raf-MEK-ERK (MAPK/ERK) pathway, is initiated. The ERK1/2 kinases are involved in the signal transduction and are co-transported with internalized NGF/TrkA or with BDNF/TrkB during retrograde transport (Delcroix et al., 2004). The ERK1/2 signaling pathway is involved in cell survival, differentiation and proliferation, but also apoptosis (Wortzel and Seger, 2011).

1.6 TREATMENT OF ALZHEIMER'S DISEASE

1.6.1 Current treatment

Although it has passed over 100 years since AD was first described, there is still no curative treatment. Researchers, patients and caregivers have a strong desire for a disease-modifying AD treatment, which we are still waiting for. The first treatments available for AD, the AChEIs, were developed in the 1990s, and are symptomatic. Interestingly, despite 30 years of intense research, still the only available medications for AD besides memantine are the AChEIs. The first in line cognitive therapy in mild-to-moderate AD is an AChEI, which reversely inhibits the AChE. The AChEIs on the market are donepezil, galantamine and rivastigmine, which are able to slightly delay further cognitive decline (Birks and Grimley Evans, 2015; Hager et al., 2014; Lee et al., 2015). In Sweden the national guidelines state that when AD is diagnosed, treatment with an AChEI is recommended (National Guidelines for the Care of Cases of Dementia. Swedish National Board of Health and Welfare 2010, Sweden, ISBN: 978- 91-86585-18-1).

Interestingly, the AChEIs have been shown to have effects on other symptoms than cognition, for example they have been found to reduce behavioral and psychological symptoms in dementia (Freund-Levi et al., 2014; Tan et al., 2020). Furthermore, the AChEIs have been associated with reduced mortality and a 35% reduced risk of death in myocardial infarction (Nordström et al., 2013) or stroke (Tan et al., 2018).

A registered drug for treatment of moderate to severe AD is the glutamate receptor inhibitor memantine. Increased amount of glutamate causes an excessive glutamate signaling via the NMDA receptor, which leads to high intracellular calcium levels and cell death. Memantine is a partial antagonist of the glutamate receptor NMDA and is used to normalize the glutamatergic signaling, stop the prolonged influx of Ca^{2+} ions, and to prevent the glutamatergic neurotoxicity (Olivares et al., 2012), but the effects of memantine in AD are considered small (McShane et al., 2019).

A meta-analysis from December 2018 found that donepezil or galantamine may show best effects on cognition in mild to moderate AD, while a combination of donepezil and memantine would be recommended for moderate to severe AD (Dou et al., 2018).

Medications to AD patients might also involve treatments for behavioral changes, psychiatric problems, sleep disturbances and depression.

1.6.2 The preclinical drug discovery process

When discovered that the effect of extracts from plants, such as morphine from opium poppy, digitalis from foxglove and salicin (ancestor to acetylsalicylic acid) from willow bark, were derived from specific chemical substances, advanced pharmacology, chemical synthesis and drug discovery was established (Jones, 2011). Generally, small molecule drugs are chemically synthesized molecules that are designed to interact with a specific target protein. Small molecule compounds and biopharmaceuticals are today the two main research areas within drug discovery. The biopharmaceutical products include vaccines, blood, tissues, gene therapies, antibodies and living cells.

Drug discovery is a long, complex and expensive process, involving both preclinical and clinical stages. The preclinical drug discovery process involves several phases including, target identification, assay development, hit identification (screening), lead identification and optimization, selection of a candidate drug (CD), safety pharmacology and toxicology as well as pharmaceutical development. Identification of a target protein is often the starting point when working with either small molecules or antibodies. The target or the receptor of interest is examined, a primary assay to study the effect of potential small molecules is developed and high-throughput screening of compound libraries are performed. Validated hits from the screen

are the starting points for chemical synthesis to optimize compounds. Structure-activity-relationship (SAR) analyses are performed to compare the chemical structure and the biological activity of the compounds. Patentability of the compounds is usually addressed early in the drug discovery process. Secondary and selectivity assays are developed, to further profile the chemical compounds. Secondary assays are used to confirm on-target effects and selectivity assays are established to avoid side effects. As more potent and selective compounds within one chemical class are identified, one or a few lead compounds are identified. A lead compound is a compound with sufficient potency and selectivity on the target of interest and with a chemical structure that makes it amenable for optimization into a CD. Thereafter, one or more lead-classes are optimized and further evaluated regarding permeability, bioavailability, toxicity, metabolism, and *in vivo* effects. The development finally reaches a point where an optimized compound fulfills all or most of the requirements, whereby a CD is selected. The compound is then ready for safety and toxicology studies in animals before pharmaceutical development and clinical trials can be initiated.

1.6.3 Future treatment

Much effort has been directed to amyloid treatment, but so far, no Aβ reducing drug has reached the market. The clinical trials with anti-amyloid antibodies are struggling with limited effects on improvement of cognition within a defined population, but also with proof-of-concept, i.e. will a reduction of Aβ in the brain cure or halt the progressive neurodegenerative decline in AD and if so, at which state of the disease must treatment start to have a disease modifying effect (Tolar et al., 2019). Two anti-amyloid antibodies are at present of particular interest, aducanumab and BAN2401 (Panza et al., 2019). Aducanumab has shown a reduction of Aβ plaques but little clinical effects and with a high frequency of amyloid-related imaging abnormalities (ARIAs). The cerebral edema related ARIAs were found in one third of patients receiving the 10 mg/kg dose compared with 10% in the placebo group. Two ongoing phase III trials with aducanumab was discontinued in March 2019 following a futility analysis, but after reanalyzing the data Biogen announced in October 2019 that they will submit aducanumab for an FDA (the U.S. Food and Drug Administration) marketing approval for the treatment of early AD (Schneider, 2020). However, recently the negative first results of two anti-amyloid drug trials (solanezumab or gantenerumab) in persons with FAD, with known mutations in the APP-gene, were reported (Washington University Press release (Feb 10, 2020), https://medicine.wustl.edu/news/alzheimers-diantu-trial-initial-results/), again dampening the enthusiasm for anti-amyloid therapies considerably.

The list of potential AD treatments is long and may involve several different target proteins and/or mechanisms. Suggested targets include Aβ clearance (Xin et al., 2018), Aβ anti-aggregation (Kocis et al., 2017), p-tau immunotherapies (Congdon and Sigurdsson, 2018), γ-secretase modulators (Aguayo-Ortiz and Dominguez, 2018), counteract the loss of tau stabilization of microtubules (Lee et al., 2011), kinase inhibitors to prevent hyperphosphorylation of tau (Annadurai et al., 2017), regulators of oxidative stress (Jiang et al., 2016), anti-inflammatory agents (Ozben and Ozben, 2019), and modulators of neurotransmitter (Kandimalla and Reddy, 2017) and neurotrophin signaling (Mitra et al., 2019; Nordvall and Forsell, 2014). While β-secretase inhibitors may no longer be a preferred therapeutic target due to target related adverse events. Another approach for fighting AD is life-style changes. Various prevention studies have shown positive signs on improved cognition in persons at risk at developing AD or dementia, among them, professor Kivipelto and co-workers have shown positive effects on cognitive in the Finnish geriatric intervention study to prevent cognitive impairment and disability (FINGER) (Kivipelto et al., 2018).

Several targets within the areas of inflammation, cholinergic signaling and neurotrophins could be amenable for pharmacological intervention and hence, potentially be of importance in future treatment of AD.

In this thesis, we focused on targeting non-amyloid pathways in AD as exemplified by developing assays to identify 15-LO-1 inhibitors to reduce oxidative stress and inflammation and by the identification of small molecular compounds that can enhance neurotrophin signaling and improve cognitive functions.

Systemic treatment with the NGF protein is associated with problems to reach the brain since BBB penetrance is very low and therapeutic doses of NGF to treat cognitive dysfunctions has been shown to cause low back pain (Eriksdotter Jonhagen, 1998). Trials with encapsulated cells delivering NGF directly to the basal forebrain of AD patients (Eriksdotter-Jönhagen et al., 2012; Eyjolfsdottir et al., 2016; Mitra et al., 2019) and treatment with gene therapy in AD patients (Rafii et al., 2018; Tuszynski et al., 2005) have both shown indications of cognitive improvement, but the invasiveness of these therapeutics are a clear drawback. When intervening with the NGF/TrkA signaling pathway to promote survival of neurons, there is a risk to enhance pain signaling. Minde and co-workers identified a single point mutation in the NGF gene (*NGF*) linked to the hereditary sensory and autonomic neuropathy type V (HSAN V) in a family in northern Sweden (Einarsdottir et al., 2004; Minde et al., 2004). HSAN V is a rare disease with a loss of pain, heat and cold sensation, where homozygote cases are more severely affected than heterozygotes. Cognition is not affected in the HSAN V patients or in

heterozygous $NGF^{R100W/wt}$ mice (Testa et al., 2019b), and the mutant NGF protein found in this disease is therefore interesting from a drug discovery point-of-view.

Small molecular treatments of both inflammation and neurotrophic signaling can be designed to cross the BBB to have effects on target cells. Inhibitors of 15-LO-1 may have the advantages to reduce inflammation without side-effects related to prostaglandin synthesis. 15-LO-1 inhibitors derived from our initiative, for example BLX3887 and BLX769, have been used to further study the 15-LO-1 activity and inflammatory responses (Archambault et al., 2018). Enhancers of NGF and BDNF signaling can be optimized to mainly strengthen TrkA and TrkB signaling in the presence of NGF or BDNF respectively, i.e. positive modulators rather than pure agonists. This would reduce the risk for peripheral signaling via NGF/TrkA which cause pain, since peripheral NGF levels normally are elevated mainly during inflammation (McMahon, 1996; Skaper, 2017). Small molecule modulators of neurotrophin receptors have been evaluated for neuroprotection, improved cognition, neurite outgrowth, cell survival and differentiation, pain reduction, healing of dry eye targeting stroke, AD, PD, traumatic brain injury, peripheral neuropathic pain, Rett syndrome, HD, epilepsy etc. (Mitra et al., 2019). The antidepressant amitriptyline has been shown to activate TrkA/TrkB and down-stream signaling events (Jang et al., 2009), but both we and others had problem reproducing these results (Boltaev et al., 2017; Todd et al., 2014). A small NGF mimetic molecule, LM11A-31-BHS, targeting the p75 receptor for the treatment of mild to moderate AD is currently in a phase IIa clinical trial, where the primary outcome is safety and secondary outcomes include Aβ, tau, AChE activity and cognitive functions, with planed completion of the study in September 2020 (James et al., 2017; Massa et al., 2006).

2 AIMS

The overall aim of this thesis was to develop assays and identify small-molecule substances to advance drug discovery projects within non-amyloid pathways in Alzheimer's disease.

Study I

The aim of this study was to develop a fluorescence-based assay, perform a high-throughput screen of a chemical library, with concomitant lead optimization, to identify small molecules inhibiting the 15-LO-1 protein.

Study II

This study aimed to elucidate the role of three nerve growth factor mutants and evaluate their ability to modify NGF/TrkA signaling.

Study III

This study aimed to screen a compound library, validate hits and to identify a potential enhancer of NGF and BDNF signaling with the ability to attenuate cognitive dysfunction.

Study IV

The aim of this study was to evaluate ACD855, as an enhancer of NGF and BDNF signaling for the treatment of Alzheimer's disease.

3 METHODOLOGY

This is a summary of the methods used in this thesis. A more detailed description of the methodological procedures can be found in the materials and methods section of each paper.

3.1 ETHICAL CONSIDERATIONS

All animal experiments were carried out in accordance with the current European Law (Directive 2010/63/EU), where all efforts were made to minimize animal suffering and to reduce the number of animals used. Experimental procedures were approved by the regional ethical board in Stockholm, the guidelines of Karolinska Institutet and the Swedish Animal Welfare Agency. Ethical permission for the use of mice and rats in animal experiments in Study III and IV were obtained by the Stockholm Regional Committees. Human fetal dorsal root ganglion (DRG) tissues were collected with informed written consent after elective routine abortions (7 to 9 weeks post conception) at Karolinska University Hospital Huddinge. All studies on human material followed the declaration of Helsinki and the guidelines of Karolinska Institutet. The permission to use fetal tissue for Study II and III was approved by the Regional Ethical Committee, Stockholm and the Swedish National Board of Health and Welfare.

3.2 MATERIALS

When developing drugs, the basis for the drug discovery is to define relevant assays that can be used as a model for the question of interest. Every model of a disease has its own advantages and disadvantages that must be considered when analyzing and interpreting data. Enzyme assays can provide the project with on-target effects, although with major limitations in the biological relevance. Overexpressing cells are biased in certain protein levels, whilst providing a model with detectable quantities of analyte in a physiological context. Primary cells represent an isolated model closer to the natural system, especially when derived from a human source. Experiments with rodents *in vivo* can provide drug discovery projects the reality of the living organism, although in a foreign species.

3.2.1 Enzyme preparation

In Study I, human eosinophils were isolated from whole blood as described before (Feltenmark et al., 2008) and RNA was prepared for further cloning, expression and purification of the 15-LO-1 enzyme.

Venous blood was collected from healthy volunteers or from patients with hyper-eosinophilia, but without hematological malignancies. Whole blood was centrifuged and applied to dextran separation. The white blood cell fraction was suspended in lysis buffer to remove contaminating red blood cells. Next, a gradient centrifugation using Lymphoprep was performed to abstract the polymorphonuclear cell fraction. Eosinophils were then isolated by negative selection using CD16 antibodies utilizing magnetic cell sorting. Purity was > 95%, tested with May-Grünwald-Giemsa solution.

Total RNA was isolated from eosinophils and human 15-LO-1 cDNA was amplified by PCR. The 15-LO-1 cDNA was cloned into the pFastBac1 vector. Bacmid DNA was derived and Sf9 insect cells were transfected. Viral particles were collected and amplified. The stock of viruses was used to infect Sf9 cells. 15-LO-1 was purified from the 20,000 g supernatant of homogenized Sf9 cells, fractions were pooled and stored at −80°C until use. The 15-LO-1 enzyme was 90-95% pure as judged by Coomassie blue staining.

3.2.2 Cell lines

Immortalized cell lines are of great value both in basic research and in drug discovery. The use of immortalized cell lines does not require an ethical permit. There are many benefits with immortalized cell lines, which usually proliferate at a high rate, survive for many passages and are often easy to culture. Cell lines can grow both as adherent cells and or in a suspension, depending on the origin of the cell type. Drawbacks with cell lines are associated with cell types that are far from the origin of the disease, cells cultured without the three-dimensional network in a normal extracellular matrix or contamination of other cell lines (Kaur and Dufour, 2012).

In Study I we used the human Hodgkin lymphoma cell line (L1236) which expresses abundant levels of 15-LO-1 (Claesson et al., 2008). The L1236 cells were used to develop cell assays amenable for studying the inhibition of the human 15-LO-1 enzyme by small molecules in a screening mode. The L1236 cell line was cultured as suspension cells.

In Study II we used rat adrenal gland pheochromocytoma (PC12) cells to study the effects of wild-type NGF and NGF mutants on cell survival and cell differentiation, and the phosphorylation potential of ERK1/2. PC12 cells are dependent on NGF for differentiation and are widely used to study neuronal changes in an immortalized cellular system (Greene and Tischler, 1976). PC12 cells are suspension cells that can be cultured as adherent cells using collagen IV coated flasks or plates. In this thesis we used adherent PC12 to study cell survival and neurite outgrowth.

Human bone osteosarcoma U2OS-TrkA/p75-SHC1 cells and U2OS-TrkB/p75-SHC1 cells, PathHunter® U2OS functional assays from DiscoverX, were used for screening and validation of small molecular compounds that can activate or enhance the neurotrophin signaling (NGF/TrkA and BDNF/TrkB). The U2OS-TrkA/p75-SHC1 and U2OS-TrkA-PLCγ1 cell assays were also used in Study II to compare the activation potential of TrkA between wild-type NGF and NGF mutants. The U2OS cells are strongly adherent cells.

3.2.3 Primary cell cultures

A primary cell culture will often proliferate less than an immortalized cell line, as primary cells commonly are of a non-tumorous origin. When preparing primary cell cultures from animal or human tissues, an ethical approval is required, and the use of these cells should always be evaluated in the light of biological and ethical relevance. Optimal growth conditions to maintain the feature of the primary cell is often critical.

In Study III we isolated primary cortical neurons (PCN) from mouse embryos on gestational day 17. PCN were used to study BDNF/TrkB mediated phosphorylation of ERK1/2 by the cardenolide AC-25793.

To study activation of TrkA we isolated human fetal DRG neurons, which are dependent on NGF for survival and differentiation. In Study II we evaluated the capacity of NGF mutants to promote cell survival, differentiation, and phosphorylation of ERK1/2, compared with wild-type NGF using DRG neurons. In Study III DRG neurons were used to evaluate the potency of AC-25793 to promote neurite outgrowth.

Cell assays		Study I	Study II	Study III	Study IV
Cell lines	L1236 cells	X			
	PC12 cells		X		
	U2OS-TrkA/p75-SHC1 cells		X	X	X
	U2OS-TrkA-PLCγ1 cells		X		
	U2OS-TrkB/p75-SHC1 cells			X	X
Primary cultures	Mouse primary cortical neurons			X	
	Human fetal dorsal root ganglion neurons		X	X	

Table 1. Cells used in experiments performed in Study I-IV.

3.2.4 Mice

Figure 5. C57BL/6J laboratory mouse.

In Study III and IV we used the C57 black 6 inbred mouse strain, originally from The Jackson Laboratory (C57BL/6J), to study *in vivo* effects of compounds that displayed potent neurotrophin enhancing effects *in vitro*. The C57BL/6J mice are the most widely used inbred laboratory mouse strain and they are well suited for behavioral testing performed with automated recording since their black coat creates a high contrast against the light background in most arenas. We used only male mice to reduce the influence by hormone cycles and the common barbering actions by the dominant female mouse, which could negatively influence the behavioral tests. C57BL/6 mice are known to be slightly more aggressive than other inbred laboratory mice, such as the Bagg albino inbred mouse strain (BALB/c) or the albino outbred mouse strain originally from Naval Medical Research Institute (NMRI) mice, but they are found attentive and respond well to behavioral testing.

3.2.5 Rats

In Study IV we used Sprague Dawley rats for LTP experiments and for microdialysis experiments, and Flinders Sensitive Line (FSL) rats in the forced swim test (FST). The FSL rats were first developed at Flinders University of South Australia through selective breeding techniques to be sensitive to the cholinesterase inhibitor diisopropyl fluorophosphate (DFP) (Overstreet and Russell, 1982). FSL rats have shown to be an animal model of depression, with high sensitivity towards behavioral treatment and changes in their cholinergic and serotonergic responses, thus responding well to cholinergic muscarinic antagonists such as scopolamine, to AChEIs such as physostigmine and donepezil and to antidepressants (Blaveri et al., 2010; Janowsky et al., 2004; Overstreet et al., 1995; Overstreet, 1993).

Animal experiments		Study I	Study II	Study III	Study IV
Mice	Hippocampal ERK1/2 analysis			X	X
	Passive avoidance			X	X
	Morris water maze				X
	Forced swim test			X	X
Rats	LTP analysis				X
	Microdialysis				X
	Forced swim test				X

Table 2. Animals used in experiments performed in Study I-IV.

3.3 METHODS

Here follows a description of the main analyses and assays used in this thesis.

3.3.1 15-Lipoxygenase-1 assays

The arachidonic acid metabolites have implication in many disorders, such as inflammation, fever, pain, coagulation, asthma as well as AD. 15-LO-1 inhibitors could be used to evaluate the inflammatory response generated by metabolites formed via the 15-LO-1 pathway in diseases such as AD. Since the human 15-LO-1 enzyme and the animal ortholog, the 12/15-LO enzyme, differ in the generated products, assay development focused on the human variant. One of the substrates of the 15-LO-1 with high Vmax is linoleic acid. Linoleic acid was used in the high-throughput screening (HTS) to obtain a robust readout and to improve the assay parameters, although focus for 15-LO-1 inhibitory treatment is the arachidonic acid metabolites.

3.3.1.1 15-LO-1 enzyme assay

An aliquot of 15-LO-1 enzyme was thawed and diluted in phosphate-buffered saline (PBS) to reach a final concentration of 2.8 μg/ml. 15-LO-1 enzyme dissolved in PBS was mixed with compound dissolved in dimethyl sulfoxide (DMSO) or DMSO as a vehicle control in assay plates and pre-incubated for 5 minutes, with a final screening compound concentration of 10 μM. Linoleic acid was added with a final concentration of 200 μM, and the mixture was incubated for another 10 minutes. The reaction was stopped with the addition of 2 volumes of methanol and 0.125 mM diphenyl-1-pyrenylphosphine (DPPP). Fluorescence was measured at 380 nm.

3.3.1.2 15-LO-1 cell assay

L1236 cells were maintained in complete RPMI (Roswell Park Memorial Institute) 1640 cell culture media. Cells were washed and diluted in PBS before seeded in assay plates. Test compound dissolved in DMSO or DMSO vehicle was added to each well and the assay plate was incubated at room temperature for 10 minutes. Linoleic acid was added, and the mixture was incubated for another 30 minutes. The reaction was stopped with the addition of methanol for high-performance liquid chromatography (HPLC) analysis or with 0.125 mM DPPP in a methanol/DMSO (1:1) mixture for fluorescence analysis.

3.3.1.3 HPLC analysis

Before reverse-phase HPLC (RP-HPLC) analysis, assay plates were stored at −20°C for at least 60 min, followed by centrifugation, to remove precipitated proteins. The supernatant was injected to a C18 column coupled to a HPLC system. Quantification of 13-hydroxy-9,11-octadecadienoic acid (13-HODE) was performed by measuring absorbance at 235 nm. Qualitative analysis was performed by comparing retention time and occasionally the UV spectra of samples with 13-HODE standard.

3.3.1.4 **Fluorescent analysis**

To quantify the levels of product formation in the enzyme or the cell assay solution, we developed a fluorescent assay that was suitable for the reactions when the 15-LO-1 enzyme catalyzes the addition of molecular oxygen into arachidonic acid or linoleic acid to form the hydroperoxides 15-HPETE or 13-HPODE, see figure 6.

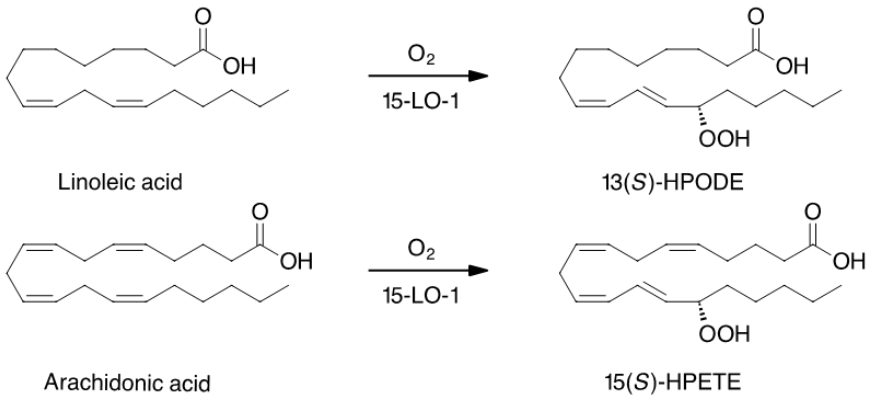

Figure 6. The oxygenation of linoleic acid and arachidonic acid by 15-LO-1 and the formation of the hydroperoxides 13-HPODE and 15-HPETE.

DPPP is a non-fluorescent compound that reacts with hydroperoxides leading to fluorescent DPPP-oxide which emit fluorescence at 380 nm. The intensity of the fluorescent light from the DPPP-oxide revealed to be proportional to the amount of 13-HODE formed in the assay, as shown in figure 7. Fluorescence was measured using a fluorescence plate reader, 15-120 minutes after the addition of DPPP + methanol/DMSO.

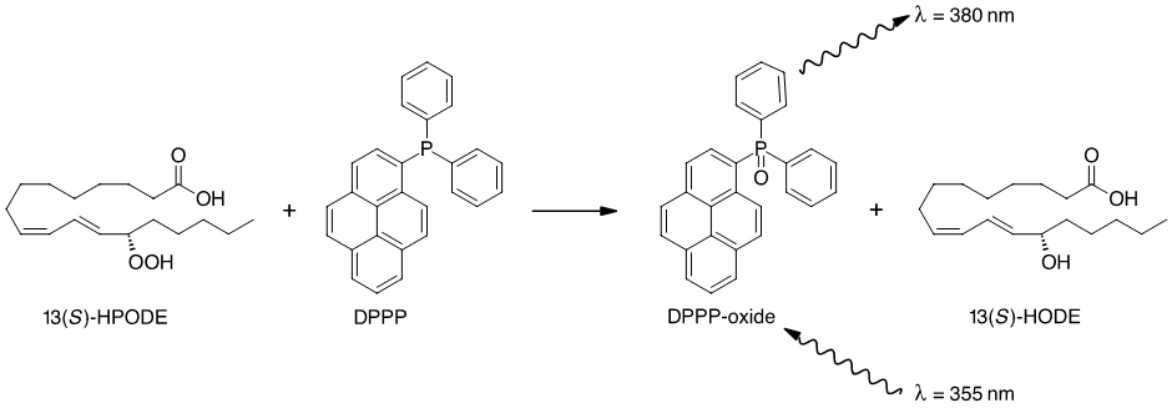

Figure 7. Reaction between the lipid hydroperoxide 13-HPODE and DPPP leading to a fluorescent DPPP-oxide.

3.3.2 Neurotrophin assays

To test the hypothesis that small molecules that enhance neurotrophin signaling can rescue degenerating neurons and improve cognitive functions in AD, the following *in vitro* and *in vivo* assays were evaluated.

3.3.2.1 PathHunter® chemiluminescence cell assay

The primary assay, used to identify and validate small molecules that modulate neurotrophin signaling in this thesis, was a PathHunter® chemiluminescence cell assay from Eurofins DiscoverX Corporation. These commercially available enginered cell assays have been validated before (Forsell et al., 2013) and for studies in thesis we used U2OS-TrkA/p75-SHC1, U2OS-TrkA-PLCγ1, and U2OS-TrkB/p75-SHC1 PathHunter® cell assays.

The U2OS-TrkA/p75-SHC1 cell assay principle is briefly described here and a simplified schematic picture is shown in figure 8. U2OS cells are stably transfected with the human TrkA receptor, which is recombinantly expressed with a small fragment of the β-galactosidase enzyme fused to TrkA at the intracellular C-terminus. The SHC1 protein is co-expressed in the U2OS cells with a larger fragment of the β-galactosidase enzyme. When the recombinant

U2OS-TrkA-SHC1 cells are treated with NGF, a dimerization and an autophosphorylation of the TrkA receptor occur. The phosphorylated TrkA receptor attracts various intracellular signaling proteins and the SHC1 protein binds the TrkA receptor at the phosphorylation site tyrosine 490 (Y490). The binding of SHC1 brings the two parts of the β-galactosidase enzyme in close proximity to each other, thereby leading to complementation which combines the two parts of β-galactosidase, generating an active β-galactosidase enzyme. After 3 hours of incubation at room temperature, a substrate is added to the assay and the active β-galactosidase enzyme converts the nonluminescent substrate to a lumiescent product. The luminescent signal detected in the assay is proportional to the level of phosphorylated TrkA receptor at the SHC1 site (Y490).

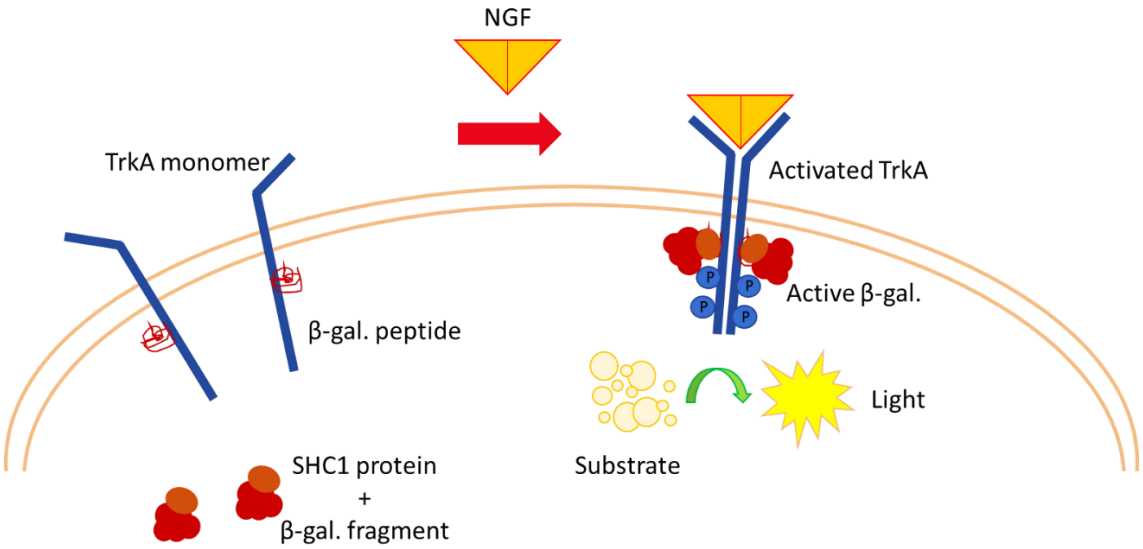

Figure 8. The enzyme fragment complementation (EFC) assay technology using β-galactosidase from Eurofins DiscoverX Corporation. This figure describes the PathHunter® functional assay with U2OS-TrkA/p75-SHC1 cells. Modified from Eurofins DiscoverX Corporation assay principle.

3.3.2.2 Phospho-TrkA and phospho-ERK1/2 ELISA

An enzyme-linked immunosorbent assay (ELISA) is a sensitive and specific method to detect proteins. The work effort is normally lower with ELISA than with for example western blot, thus the number of analyzes can be increased. To quantify the levels of phosphorylated TrkA or ERK1/2 in cell suspension, we used the human phospho-TrkA DuoSet IC ELISA and phospho-ERK1 (T202/Y204)/ERK2 (T185/Y187) DuoSet IC ELISA, both from R&D Systems, according to the manufacturer's instructions.

3.3.2.3 Western blot analysis

Western blot is an immune-based method to detect proteins by size and it is a sensitive and a specific method to detect and quantify proteins. In Study II, III and IV, western blot analysis was used to quantify and compare protein levels in cultured cells or mouse brain tissue.

Briefly, the cells were lysed, or the mouse hippocampal tissue was sonicated, in standard lysis buffer complemented with protease and phosphatase inhibitors. Samples were mixed with 4X loading buffer, heated to 95°C for 5 minutes and loaded on Novex Bis-Tris 4-12% gels for protein separation. A dry gel transfer to polyvinylidene difluoride (PVDF) membrane was performed using an iBlot instrument. Membranes were blocked in a Tris-buffered saline (TBS) solution supplemented with 0.1% Tween and 5% nonfat dry milk or bovine serum albumin (BSA). Primary and secondary anti-bodies were diluted in a TBS-Tween solution supplemented with 5% BSA. Primary antibody phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) rabbit monoclonal IgG1 antibody was from Cell Signaling. Secondary antibody was a horseradish peroxidase-coupled anti-rabbit antibody. Quantification was performed using Image Studio Lite.

3.3.2.4 Immunocytochemistry analysis

The immunocytochemistry analysis was utilized to examine NGF dependent proliferation and differentiation, and to study TrkA down-stream signaling. Compared to other immunoassays, immunocytochemistry assays allow for studies of protein expression at a cellular or subcellular level. This study tested the hypothesis that mutant NGF might affect cell survival, neurite outgrowth and activation of ERK1/2 differently, or that small molecules enhancing the NGF/TrkA signaling could potentiate neurite outgrowth in combination with NGF. Beta-tubulin III antibody detects a specific tubulin found only in neurons (and testis cells), and make it possible to study neurite length, neurite branching, neurite width and other parameters linked to individual neurons. Secondary antibodies conjugated with Alexa Fluor dyes were used to visualize detected proteins and automated fluorescence imaging (Thermo Scientific Cellomics Array Scan VTI HCS Reader) was used to quantify the fluorescent signal. Automated examination of captured images was performed using a high-content imaging analysis software (Cellomics).

Human fetal DRG tissue was collected and dissections were performed under sterile conditions, tissue collected in DMEM medium and disassociation of ganglia to isolate single cells started within one hour. Ganglion tissue was dissociated using TrypLE at 37°C for 4 minutes and the reaction was inhibited using complete cell media, centrifuged to remove debris and repeated twice. DRG cells were counted using a Bürker chamber and diluted in Neurobasal cell culture

media supplemented with 2% B27, 0.5 mM L-glutamine and penicillin/streptomycin. DRG cells were seeded in black 384-well view plates with clear bottom from Perkin Elmer. Assay plates pre-coated with poly-D-lysine (PDL) were coated with 0.01% fibronectin and dried in the hood just before plating of cells.

PC12 cells from ATCC were cultured adherent in collagen IV coated flasks in DMEM medium supplemented with 10% FBS, 5% horse serum and penicillin-streptomycin. Before assay, PC12 cells were diluted in DMEM medium supplemented with 1% horse serum and penicillin-streptomycin to support NGF dependent differentiation, and seeded in black 384-well view plates, pre-coated with PDL and coated with collagen IV.

DRG neurons or PC12 cells were treated with various concentrations of NGF (27 kDa, PeproTech, Rocky Hill, NJ, USA), mutant NGF (Acturum, Södertälje, Sweden), or compound for 2-8 days, according to the protocol in each study. Cells were incubated at 37°C in a humidified atmosphere with 5% CO_2 without any change of media until fixed in 4% (v/v) formaldehyde solution. Cells were permeabilized by incubation in 0.3% Triton X-100 in TBS and blocked with 2.5% BSA in 0.3% Triton X-100 in TBS to reduce non-specific binding for one hour at room temperature. Primary antibody incubations were performed in blocking solution overnight at 4ºC. Primary mouse anti-β-tubulin antibody was used to detect microtubules found along the neurites. Primary rabbit anti-phospho-p44/42 (pERK1/2) antibody was used to study activation of the ERK1/2 protein, a presumable TrkA or TrkB down-stream signaling event linked to cell proliferation, survival and differentiation, and functions in learning and memory (Peng et al., 2010; Zhou et al., 2014).

Secondary antibody incubations were performed using goat anti-mouse Alexa-488 or goat anti-rabbit Alexa-647, incubated together with Hoechst nuclear stain, in blocking solution. Cellular nuclei were quantified using Hoechst and cells were defined as β-tubulin positive objects with nucleus and cell soma, and neurite outgrowth was defined as average neurite length per valid cell in micrometer. Objective automated imaging analysis ensured unbiased quantification and results were exported as mean values per well.

3.3.2.5 Hippocampal activation of ERK1/2

Memory problems, learning difficulties and cognitive dysfunctions are shown to be associated with dysfunctional BDNF/TrkB signaling and hippocampal alterations, therefore the activation of the down-stream signaling protein ERK1/2 was analyzed in the mouse hippocampus after compound treatment.

Male C57BL/6J mice received subcutaneous (s.c.) injections, 1 mg/kg/day of AC-25793 or PBS vehicle, for five consecutive days. 3 hours after the last injection, animals were

decapitated, and hippocampus was dissected. Quantification of phospho-ERK1/2 levels in the hippocampus were performed by western blot analysis.

3.3.2.6 Long-term potentiation

Hippocampal LTP is the cellular mechanism for memory formation induced by glutamate release which has been shown to be potentiated by BDNF (Abrahamsson et al., 2016; Woo and Lu, 2009). Electrophysiological LTP studies were performed as described earlier (Abrahamsson et al., 2016; Ho et al., 2011). Briefly, Sprague Dawley male rats were deeply anesthetized using isoflurane, brains were removed, and horizontal hippocampal sections were prepared to maintain the connections in the Shaffer collaterals (SC)/CA1 pathway (Fig. 9). After slicing at 34°C, slices were cooled down to room temperature and then allowed to recover for 2 hours. Slices were transferred to submerged recording chambers, perfused with in-house prepared artificial cerebrospinal fluid (aCSF) at 32°C and bubbled with carbogen gas (5% CO_2, 95% O_2). Field excitatory postsynaptic potentials (fEPSPs) were recorded using standard electrophysiological equipment. Presynaptic stimulation was applied in the SC (CA3 region) and postsynaptic signals were recorded in stratum radiatum (CA1 region), both the stimulatory electrode and the recording pipette were placed extracellularly.

After 30 minutes of baseline recordings, a subthreshold theta-burst (θ-burst) stimulation was delivered to the SC. The normalized fEPSP slope was calculated after recordings in the CA1 region of control slices treated with aCSF which were compared with slices treated with BDNF (50 ng/ml) or ACD855 (20 µM).

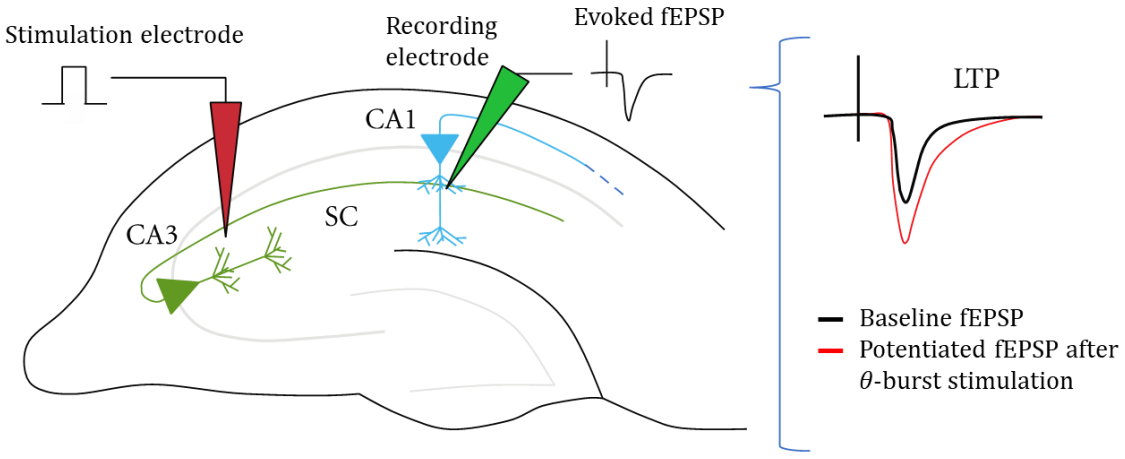

Figure 9. LTP experiment in rat hippocampal slices. Modified from Cramer *et al*. (Cramer and Galdzicki, 2012).

3.3.2.7 Microdialysis

To elucidate the biochemical effects of ACD855 on neurotransmitter release in the brain of rats, a microdialysis experiment was performed. A small microdialysis probe was placed in the ventral hippocampus and inserted using a guide cannula on an awake rat, see figure 10.

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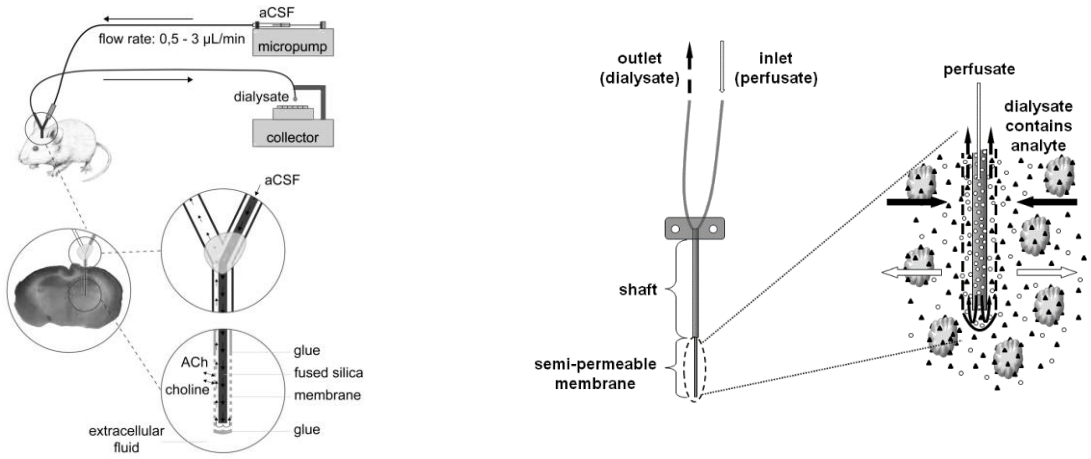

Figure 10. (A) The principle of the microdialysis procedure (König et al., 2018). (B) Schematic illustration of a microdialysis probe (Wikipedia).

The probe was perfused at a constant flow-rate with aCSF solution and baseline levels of neurotransmitters were recorded for 4 hours, followed by perfusion with 30 µM ACD855 dissolved in aCSF solution while collecting samples for another 4 hours. The microdialysis experiment was finalized with a wash-out period for 2 hours with aCSF solution and sampling. Animals were sacrificed by an overdose of isoflurane and neck dislocation. The brains were removed, frozen on dry ice and stored at -80°C for biomarker analysis or histological verification.

3.3.3 Behavioral tests

The rationale for testing the compounds in behavioral tests evolved from potent enhancing effects on the TrkA and the TrkB receptor *in vitro* in combination with a subset of down-stream signaling events, neurite outgrowth abilities, and hippocampal *in vivo* studies in rodents.

3.3.3.1 Morris water maze

The Morris water maze is a test for spatial learning, where the animal is placed in a large water tank (180 cm wide, 45 cm deep, filled to 28 cm with 23°C tap water) and seeks a hidden

platform (10 cm wide, 1 cm below water surface) with the help from distal ques (Kuteeva et al., 2005; Morris et al., 1982). The procedure is repeated several times and an animal with good cognitive abilities will find the hidden platform more rapidly after each trial, while an animal with dysfunctional cognitive abilities will continue to search for the platform without improved routes. The swimming sessions were video recorded, and the swimming patterns analyzed.

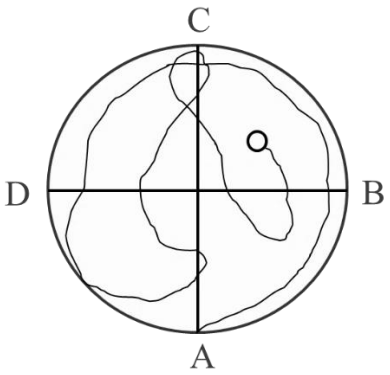

Figure 11. Example of a recorded swimming trajectory after a training trail in the Morris water maze with a hidden platform.

Compound (ACD855, 3 mg/kg) or vehicle (20% DMSO in 0.1 M sodium phosphate buffer pH 8.0) were injected s.c. for 10 consecutive days. Behavioral testing was performed day 5-10 with additional injections of scopolamine (0.3 mg/kg) or vehicle (saline) during the days of behavioral training. Scopolamine causes a transient memory deficiency with a reduced ACh signaling due to blocking of muscarinic receptors (Bartus, 2000; Bymaster et al., 1993). The time to find the hidden plat form (swim latency), swim speed, probe trial (where plat form is removed) and time spent in target quadrant (where the hidden plats form was situated), were analyzed. The experiment was divided into two sessions to be able to perform the experiments at approximately the same time of the day for all animals. Each session included 16 animals and unpaired t-tests were used for groupwise comparisons to ensure non-significant differences between sessions.

3.3.3.2 Step-through passive avoidance task

The passive avoidance task is a test used to study memory and cognition in rodents. The passive avoidance model has been refined and optimized by our group (Madjid et al., 2005). The passive avoidance test is based on an aversive stimulus, such as the short and weak, but slightly painful foot-shock delivered through the stainless-steel bar floor in the dark and preferred area

of the test case. The test chamber is divided into a bright and a dark compartment, with an automatic door between the two chambers. Before the first trial, the animals were injected with test compound (AC-25793 or ACD855) or vehicle (PBS for AC-25793 or 20% DMSO in 0.1 M sodium phosphate buffer pH 8.0 for ACD855) for 4 consecutive days according to the protocol in each study. 30 minutes before the training session day 4, animals were also injected with a single dose of scopolamine (0.3 mg/kg) or MK-801 (0.3 mg/kg) to induce cognitive dysfunction in selected groups according to protocol in each study. MK-801 cause a memory deficiency due to NMDA receptor blocking. A single dose of the TrkB inhibitor ANA-12 (0.5 mg/kg) was injected to study the BDNF/TrkB dependent effect of ACD855 in the passive avoidance model. A single low dose of 0.025 mg/kg physostigmine was injected to study the additive effect on a sub-effective dose of ACD855 (1 mg/kg). On the training day, the animal was placed in the bright compartment with the door closed and when the door was opened after 1 minute, the animal searched for protection in the dark compartment, where it got a mild foot-shock (0.35-0.4 mA, for 2 seconds, scrambled current). The time to enter the dark compartment on the training day is referred to as the training latency. 24 hours later (test day), the animal was reassembled to the bright compartment and when the door was opened, the time for the animal to enter the dark compartment was measured, referred to as the retention latency. Animals with normal learning and memory will avoid entering the dark chamber on the test day, where it received a foot-shock.

To be able to perform the experiments at approximately the same time of the day for all animals, the experiment with AC-25793 was divided into two sessions. Unpaired t-tests were used for groupwise comparisons to analyze if the animals performed differently in session one and session two, which was not the case and therefore the two sessions were analyzed as one single experiment.

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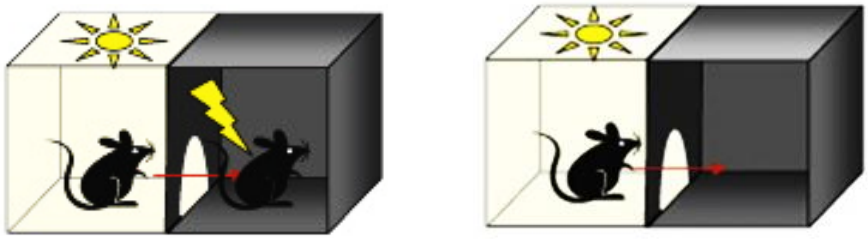

Figure 12. The passive avoidance paradigm. A.) Training in shuttle-box with a mild foot-shock day one. B.) Recording of time to enter (retention latency) the dark compartment day two.

Passive avoidance		Day				
		1	2	3	4	5
Actions	AC-25793 or ACD855 injections	X	X	X	X	
	Physostigmine or ANA-12 injection				X	
	Scopolamine or MK-801 injection				X	
	Training session				X	
	Retention test					X

Table 3. Experimental design of the passive avoidance experiment presented in Study III-IV.

3.3.3.3 Forced swim test

In addition to its connection to synaptic plasticity and memory formation, BDNF has been shown to be associated with depression and mood disorders. Depression, depressive symptoms and use of antidepressants are common in AD (Chi et al., 2014; Enache et al., 2016). Following the cognitive evaluations with the neurotrophin enhancing compounds, the FST, a test for depression-like symptoms in mice or rats, was performed. The FST was developed by Porsolt (Porsolt et al., 1977) and the test used in this thesis was modified by Kuteeva *et al* (Kuteeva et al., 2005).

The FST uses a cylinder, half filled with lukewarm water. The size of the container and the water level is chosen to avoid that the animal can climb out of the water filled container or touch the bottom. Injections of test compound (AC-25793 or ACD855) or vehicle (PBS for AC-25793 or 20% DMSO in 0.1 M sodium phosphate buffer pH 8.0 for ACD855) were given s.c. for five consecutive days and injections of reference compound (fluoxetine) or saline vehicle was given s.c. as a single administration 30 minutes before the FST day 5. A 10 minutes pre-swimming session was used to build up a stressor, which has been shown to evoke a depression-like behavior, and the test was performed for 6 minutes the following day. When the animal was placed in the cylinder, it immediately started intense swimming (Fig. 13A).

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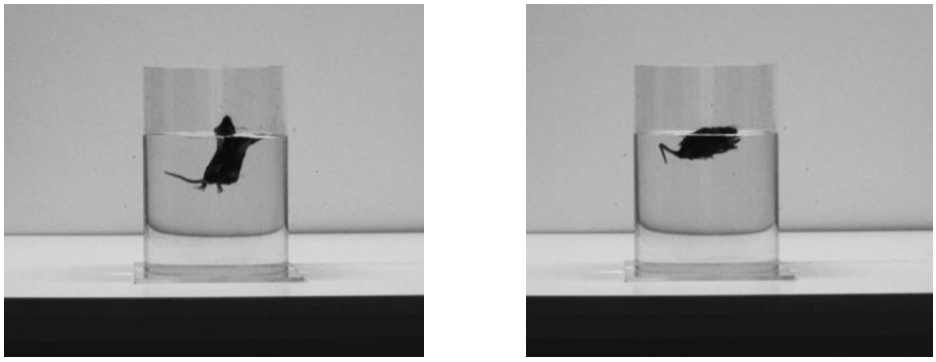

Figure 13. C57BL/6J mice in the forced swim test. (A) Intense swimming. (B) Immobile floating behavior.

On the test day, shortly after the animal was placed in the water tank, it gave up swimming and exhibited a distinctive immobility (Fig. 13B). The animals altered between swimming and floating during the 6 minutes task, with more and more floating towards the end of the test. The immobility time is considered an index of depression-like behavior and is defined as the time spent floating with maximum one paw moving to keep the balance. The swimming and immobility behaviors were monitored, and either video recorded or measured directly.

Forced swim test		Day				
		1	2	3	4	5
Actions	AC-25793 or ACD855 injections	X	X	X	X	X
	Fluoxetine injection					X
	Training session				X	
	Swim test					X

Table 4. Experimental design of the forced swim test in Study III-IV.

3.3.4 Statistical analysis

The software GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA), was used for all statistical calculations. Half maximal effective concentrations (EC_{50} values) and half maximal inhibitory concentration (IC_{50} values) was calculated using non-linear regression analysis. An unpaired t-test was used to compare two groups. To test whether dose-response curves differed from each other, non-linear regression with extra sum-of-squares F test was used. The overall treatment effects were analyzed using a one-way analysis of variance (one-way ANOVA) for all experiments with more than two groups, except for the Morris water maze, which was analyzed using a two-way ANOVA with repeated measurements. If significant, Bonferroni or Tukey's multiple comparison tests were performed to assess statistical difference between groups. The level of significance was set at 0.05.

4 RESULTS AND DISCUSSION

In this section, the main findings from the studies included in this thesis are summarized and discussed. More detailed results are found in each study. The main aim in this thesis was to advance drug discovery projects within non-amyloid pathways in AD and two non-amyloidogenic approaches for treatment of AD have been studied.

1. Inflammation, reactive oxygen species and 15-LO-1: Knowledge on the function of the 15-LO-1 enzyme in relation to drug development in AD is limited. The development in Study I of a high-throughput screening assay and the indentification of potent 15-LO-1 inhibitors enables the evaluation of small molecules affecting the 15-LO-1 enzyme activity in inflammatory diseases such as AD.

2. Neurodegeneration, cognitive dysfunction and neurotrophins: The involvement of the neurotrophins in neurodegeneration, AD and memory impairment is well known but more detailed knowledge on different types of NGF and effects on NGF and BDNF signaling is needed. Study II evaluates NGF mutants and their ability to affect TrkA signaling and neurite outgrowth abilities. Study III and IV describe drug discovery and identification of two small molecules that potentiate the NGF/TrkA and the BDNF/TrkB signaling with positive effects in behavioral models of cognition and depression.

4.1 STUDY I

Development of a fluorescent intensity assay amenable for high-throughput screening for determining 15-lipoxygenase activity

Arachidonic acid and its metabolites are key players in the immune response, with both pro- and anti-inflammatory actions. Elevated levels of 15-LO-1 is found in airway tissues of patients with asthma, in certain cancers and in affected brain regions in AD (Mattoli et al., 1990; Praticò et al., 2004).

Several studies have shown the involvement of 15-LO in AD or AD-like animal models. Increased levels of 15-HETE has been shown in CSF and in frontal and temporal regions of the brain in AD patients (Praticò et al., 2004; Yao et al., 2005). Reduction of the animal ortholog 12/15-LO has shown improvement of memory deficits, reduced Aβ pathology and improved tau pathology in the AD-like transgenic mouse model (Chu et al., 2015; Joshi et al.,

2015). But, the lack of potent and selective 15-lipoxygenase inhibitors has hampered the pharmacological validation of 15-LO-1.

To identify inhibitors of 15-LO-1 a high-throughput screen was performed. This study started with assay development and small-scale screening of potential 15-LO-1 inhibitors. Early in the project the Hodgkin Reed-Sternberg L1236 cell line was identified as a suitable cell assay for the project with high endogenous levels of the 15-LO-1 protein (Claesson et al., 2008). An enzyme assay with purified human 15-LO-1 enzyme and a cell assay using the L1236 cells were developed and implemented in parallel. Chemical synthesis, SAR analysis and structure development were performed by the medicinal chemistry team members.

Both the 15-LO-1 enzyme assay and the L1236 cell assay were examined using HPLC analysis. With higher demands on compound testing and approaching an HTS campaign, the need for a cheaper, faster and less labor-intensive analysis method was of great interest. Many HTS assays use absorbance or fluorescent signals to detect the production or the disappearance of a product or substrate in the assay, which is shown to be a stable and efficient analysis method. In this project, we developed for the first time a screening assay using the DPPP compound with a fluorescence analysis method. The lipid hydroperoxides, 15-HPETE or 13-HPODE, formed when 15-LO-1 catalyzes the peroxidation of arachidonic acid or linoleic acid were evaluated to directly oxidize the non-fluorescent DPPP compound in a one-step reaction by forming DPPP-oxide. By quantifying the fluorescence at 380 nm, we could indirectly measure the formation of the 15-LO-1 hydroperoxide metabolites.

To optimize the assay, we decided to use the substrate with the highest 15-LO-1 catalytic efficiency, which was linoleic acid. The assay involved the addition of linoleic acid to the 15-LO-1 enzyme mixture or the cell suspension to initiate the reaction. The reaction was stopped by the addition of methanol or methanol containing 0.125 mM DPPP. Analysis of formed 13-HPODE/13-HODE was performed using HPLC or fluorescence.

Except for the transformation from HPLC analysis to fluorescence analysis, the assay was also transferred from a 96-well format to a 384-well format. This mainly cuts the cost, since the number of plates, amount of enzyme or cells, and chemicals can be reduced. In an HTS setting, automatization is important to reduce the labor intensity and to minimize human mistakes. The HTS assay developed in this study was automated using a Biomek NX robot. However, aspiration and dispensing of DPPP dissolved in pure methanol encountered difficulties with the surface tension using the 384-channel robotic head. To circumvent these problems, DPPP was dissolved in a mixture of methanol and DMSO (1:1 ratio). The background fluorescence increased using DMSO in the stop solution, which caused a slight decline in assay quality. Both signal-to-background (S/B) ration and Z-prime factor (Z') values decreased with DMSO in the

solvent solution. Z' factor was still above 0.4 in the fluorescent assay, which is set as a common benchmark in biological assays (Table 5).

Table 1. Summary of Assay Parameters

Detection Format	*Format*	*Type of Assay*	*Plates, n*	*Signal to Background, Mean ± SD*	*Z', Mean ± SD*
Fluorescence	96	Biochemical	60	11.3 ± 2.8	0.79 ± 0.10
Fluorescence	96	Biochemical	565	10.9 ± 2.7	0.77 ± 0.13
Fluorescence	384	Biochemical	130	2.9 ± 0.4	0.40 ± 0.13
High-performance liquid chromatography	96	Cell	44	15.2 ± 5.9	0.69 ± 0.16
Fluorescence	384	Cell	6	3.0 ± 0.4	0.46 ± 0.05

Table 5. Summary of assay parameters from the 15-LO-1 HTS assay development (Dahlström et al., 2010).

The quality of the assays were carefully monitored during assay development (Zhang et al., 1999). S/B, coefficient of variation (%CV) and Z' factor were calculated according to following equations:

$$\mathrm{S/B} = \frac{\mathrm{MEAN\ max}}{\mathrm{MEAN\ min}}$$

$$\%\mathrm{CV} = \frac{\mathrm{SD\ max}}{\mathrm{MEAN\ max}} * 100$$

$$\mathrm{Z'\ factor} = 1 - \frac{(3\ \mathrm{x\ SD\ max} + 3\ \mathrm{x\ SD\ min})}{|\mathrm{MEAN\ max} - \mathrm{MEAN\ min}|}$$

S/B = Signal-to-background

%CV = Coefficient of variation

SD = Standard deviation

max = max controls = absence of inhibitor

min = min controls = absence of 15-LO-1 enzyme or with 2 µM of known inhibitor

The fluorescent assay for the quantification of 15-LO-1 activity was developed using the non-fluorescent DPPP compound which is oxidized to the fluorescent DPPP-oxide by the hydroperoxide formed when 15-LO-1 catalyzes a polyunsaturated fatty acid. The formation of DPPP-oxide was found to be linear with the product formation of AA or LA metabolites within the defined concentration interval used. Since the reaction can be carried out at room temperature and the DPPP-oxide is stable for up to 2 hours, it is a convenient method and well suitable for HTS campaigns, where several plates can be analyzed consecutively.

The assays were suitable for both enzyme and cell analyzes and could be transformed from a 96-well format to a 384-well format. Using this assay, several potent 15-LO-1 inhibitors such as BLX-000769 (also known as BLX769) were identified. BLX769 has been further investigated by others. Recently, Archambault and colleagues showed that BLX769 is a potent 15-LO-1 inhibitor in human eosinophils, one of the key effectors of inflammatory responses (Archambault et al., 2018) giving further support to the 15-LO involvement in inflammation.

There are of course limitations as in all studies. In Study I, a methodological limitation was that also 5-LO inhibitors were identified as positive inhibitors, which could be solved by a 5-LO selectivity assay. Another limitation was the aspiration and dispensing of DPPP dissolved in pure methanol using the 384-channel robotic head, which encountered difficulties with the surface tension. To circumvent these problems, DPPP was dissolved in a mixture of methanol and DMSO (1:1 ratio). The background fluorescence increased using DMSO in the stop solution, which caused a decline in assay quality. Eventually could this problem be solved by adding the stop solution using a multi-drop instead of a 384-channel robotic head.

A possible limitation to this study would be that inhibition of the 15-LO-1 protein might be beneficial or disadvantageous at different stages of the disease progression. Another relevant question is if the inhibition of 15-LO-1 in AD provide a window-of-opportunity where treatment effects will be beneficial. Further studies are clearly needed on the effect of 15-LO-1 on inflammation in AD.

In summary, a human 15-LO-1 cell and enzyme assay amenable for HTS screening of small molecular compounds, with a new application of DPPP was developed. The assay is robust and accurately targets 15-LO-1 activity. The identified inhibitors of the 15-LO-1 can then be used to evaluate the involvement of the 15-LO-1 enzyme in various disorders, such as AD.

4.2 STUDY II

Identification of amino acid residues of nerve growth factor important for neurite outgrowth in human dorsal root ganglion neurons

NGF signaling is found important for cell survival, differentiation and proliferation, and BFCN degeneration is linked to reduced NGF signaling via TrkA and AD pathology. A small molecular treatment designed to enhance NGF/TrkA signaling is a suggested target for the treatment of AD, while there is a risk to enhance pain signaling which was shown with intracerebroventricular infusion of NGF to three AD patients (Eriksdotter Jonhagen, 1998). This pain eliciting response with intracerebroventricular delivery may be caused by affecting the DRG and hypothalamus function (McKelvey et al., 2013). Although, this pain signaling effect was not found when NGF was administered directly into the brain parenchyma in rats (Hao et al., 2000) or in individuals with AD (Eriksdotter-Jönhagen et al., 2012; Eyjolfsdottir et al., 2016; Tuszynski et al., 2005).

To further investigate NGF/TrkA and its down-stream signaling, we performed a study with NGF mutants. Professor Cattaneo and colleagues have coined the phrase "painless" NGF with respect to the HSAN V mutant NGF-R100W and its potential as a neuroprotective treatment without the pain side-effects (Capsoni et al., 2017, 2012, 2011b; Cattaneo and Capsoni, 2019; Covaceuszach et al., 2010; Malerba et al., 2015). Both the Italian group and our group have studied a variant of the HSAN V mutant, NGF-R100E, with equivalent properties to the naturally occurring mutant, NGF-R100W (Capsoni et al., 2011b). In our study we also included two additional mutants, an NGF mutant related to the lipid binding site on NGF in mast cells, NGF-W99A (Tong et al., 2012) and a double mutant related to neurite outgrowth properties, NGF-K95A/Q96A. The mutated amino acids, 95, 96, 99 and 100, are in close proximity to each other and are depicted in figure 14.

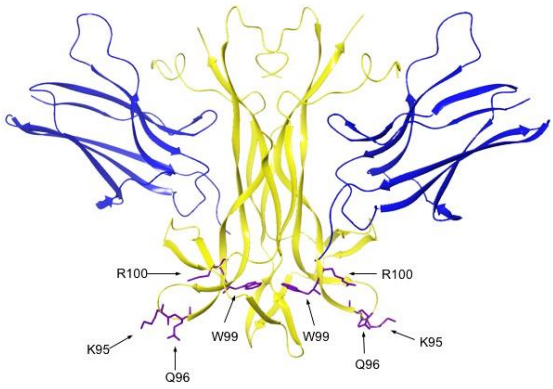

Figure 14. An X-ray structure of the interacting part of the NGF-dimer in yellow and the extracellular D5 domain of the TrkA receptor in blue (Dahlström et al., 2019).

In Study II, the activation of the TrkA receptor at tyrosine 490 (the SHC1 binding site) and at tyrosine 785 (the PLCγ1 binding site) was examined in U2OS-TrkA/p75-SHC1 cells or U2OS-TrkA-PLCγ1 cells using the PathHunter® assay described above, where we compared wild-type NGF with the three NGF mutants, R100E, W99A and K95A/Q96A. In addition, cell survival, neurite outgrowth and ERK1/2 activation were studied in human DRG neurons and in PC12 cells. We found that NGF-W99A and NGF-K95A/Q96A displayed lower potency to induce TrkA and ERK1/2 signaling, and a decreased ability to induce cell survival and neurite outgrowth compared with wild-type NGF. These results indicate that amino acid 95, 96 and 99 in the NGF protein are important for TrkA signaling and down-stream events.

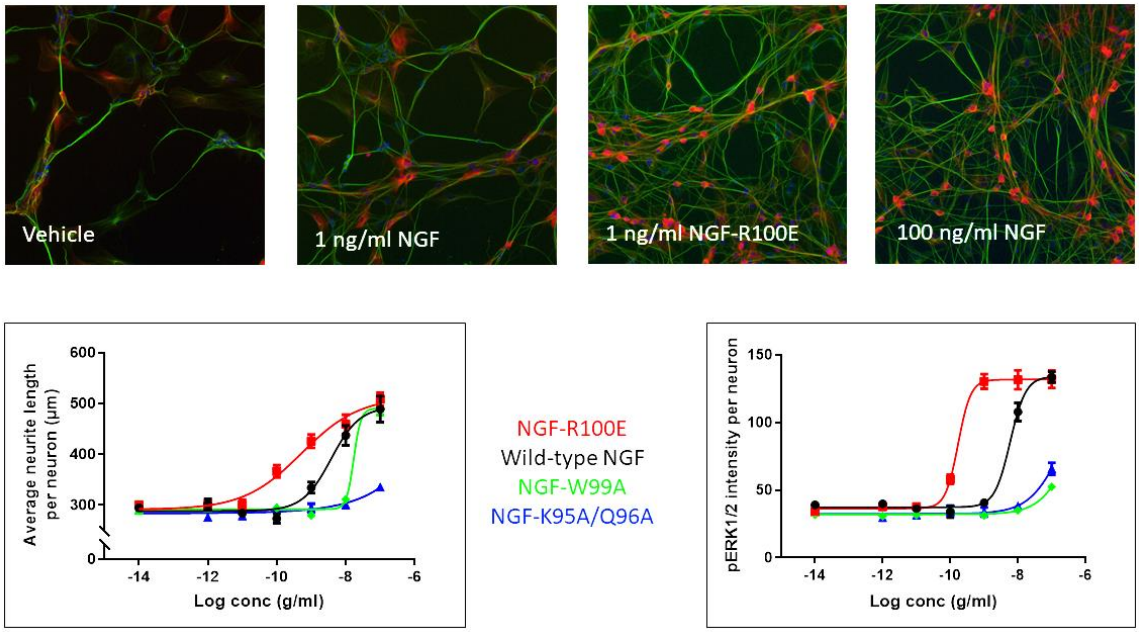

Figure 15. Analysis of human fetal cultured DRG neurons treated with NGF, mutant NGF or vehicle. Cultures were stained with Hoechst nuclear stain to label cell nuclei (blue), anti-β-tubulin antibody to label cell bodies and neurites (green) and anti-phospho-pERK1/2 (red).

On the contrary, the NGF-R100E mutant displayed potent effects on TrkA signaling and the examined down-stream events. We showed that the R100E mutant was more potent than both wild-type NGF, W99A and K95A/Q96A regarding activation of TrkA at the SHC1- and the PLCγ1-site in recombinant cells, and cell survival, neurite outgrowth and ERK1/2 phosphorylation in human DRG neurons (Fig. 15). The potent activation of NGF-R100E mutant at the TrkA-PLCγ1 phosphorylation site (Y785) in U2OS-TrkA/p75-SHC1 cells was somewhat surprising to us, since others had found reduced activation at this epitope of the NGF-R100E mutant compared to wild-type NGF in BALB/C 3T3 TrkA cells and in PC12

(Capsoni et al., 2011b). Albeit, we also found a reduced activity of NGF-R100E in PC12 cells at shorter incubation times (2-4 days), when studying cell survival and neurite outgrowth, and we could show that this effect was time dependent with increased potency after 6-8 days in culture. Our findings suggest that the choice of cell type and the incubation time may have substantial effects on the results.

Our study demonstrates that the HSAN V-like NGF mutant NGF-R100E is a potent TrkA-signaling enhancer, with superior cell survival and neurite outgrowth abilities compared with the wild-type NGF in human fetal DRG neurons. The NGF-R100E and the natural mutant NGF-R100W have been previously reported to have maintained binding affinity for the TrkA receptor and less binding affinity for the p75 receptor (Covaceuszach et al., 2010). It has also been shown in a homozygous $NGF^{R100W/R100W}$ mouse model the that number of cholinergic neurons are equally high in the basal forebrain and even increased in the striatum compared with wild-type mice (Testa et al., 2019a).

The access to human fetal DRG neurons is a rate limiting step during assay development and in the number of experiments that can be performed, but we found it worth the effort to be able to study a human primary neuronal cell culture which is dependent on NGF for survival and differentiation. The ethical perspective of using human fetal tissue must be evaluated from study to study. Prior to an abortion, the woman is offered to donate fetal tissue for research, but she is under no pressure to donate and will receive equally good medical care whether she donates tissue or not. The use of human material is highly valuable for future treatments in neurodegenerative diseases.

In summary, the combination with potent TrkA-signaling and subsequent neuronal protection and low pain signaling for the R100-mutants, offers a unique target for drug discovery of small molecular compounds, or as a protein treatment if administered locally.

4.3 STUDY III

Steroid derivative AC-25793 enhances neurotrophin signaling and attenuates cognitive dysfunction in mice

The cholinergic hypothesis suggests that short-term memory problems in AD are linked to decreased NGF signaling, which may cause degeneration of BFCN, and thus contributes to insufficient signaling of ACh (Craig et al., 2011). Long-term memory is formed and processed in the hippocampus. The cognitive dysfunctions and the long-term memory problems found in AD have been reported to be linked to hippocampal degeneration and BDNF deficiency (Miranda et al., 2019).

To address the cognitive dysfunction associated with reduced neurotrophic support, we aimed to identify small molecules that could enhance TrkA and TrkB signaling to attenuate cognitive dysfunctions. A U2OS-TrkA cell-based assay was used, as described in section 3.3.2.1, and a high-through put screen of 25,000 compounds was performed. After validation of hits and additional screening of small focused libraries, a class of potent TrkA activators with a steroid backbone emerged. It has been shown before that steroid derivatives can improve cognition (Laudisio et al., 2009; Tohda et al., 2012) and we found the relationship with neurotrophin modulatory effects of interest and worth pursuing.

Dose response curves for 88 steroid derivatives were evaluated using the U2OS-TrkA/p75-SHC1 cell assay at 10 ng/ml NGF, thus studying TrkA phosphorylation at tyrosine 490 (Y490). Sixteen of these steroid derivatives displayed an enhanced signaling of TrkA with an EC_{50} value below 500 nM. The most promising candidate for further evaluation, AC-25793, was selected based on its potent enhancement of TrkA and TrkB signaling and its good solubility in PBS.

The structure of AC-25793 contains a cardenolide component and NMR studies revealed that AC-25793 and k-strophanthidin have all the stereocenters in the same configuration (Fig. 16).

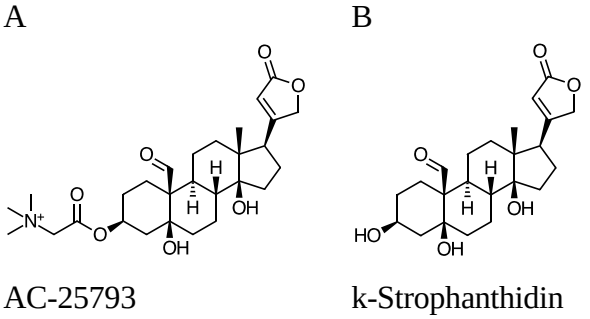

Figure 16. Chemical structure of the cardenolides (A) AC-25793 and (B) k-strophanthidin. The cardenolide structure is defined by the lactone ring (cyclic carboxylic ester) linked to the steroid backbone.

With its quaternary nitrogen, AC-25793 is more water soluble than k-strophanthidin but less expected to pass the blood-brain barrier. The ester bond between the quaternary nitrogen and the cardenolide ring system is most likely susceptible to plasma hydrolysis and AC-25793 can thereby act as a soluble pro-drug to k-strophanthidin *in vivo*.

In Study III we found that incubation of cells with AC-25793 led to increased phosphorylation of both the TrkA and the TrkB receptor, as assessed by potent enhancement of the signaling in U2OS-TrkA and U2OS-TrkB cells with EC_{50} values of 25 or 36 nM, respectively. AC-25793 could activate down-stream events, demonstrated by the potentiation of NGF dependent neurite outgrowth in human fetal DRG neurons and potentiation of BDNF dependent ERK1/2 phosphorylation in mouse primary cortical neurons. Also, injections of 1 mg/kg/day of AC-25793 for five consecutive days induced activation of ERK1/2 in the mouse hippocampus (without behavioral testing). Down-stream signaling of the Trk receptors may activate ERK1/2, which has been demonstrated to be critical for LTP, cell survival and cell differentiation (Lu and Xu, 2006; Marshall, 1995; Schafe et al., 2008).

Based upon the potent effects on NGF- and BDNF-signaling in our examined models described above, we initiated behavioral studies with AC-25793. We started with s.c. injections of 3 or 10 mg/kg in a few C57BL/6 male mice. Both these doses induced sedation and withdrawal a few minutes after compound injection and lasted for about 30 minutes, with less adverse effects in the lower dose tested. One hour after the injection, all mice displayed normal behavior again. Due to these transient adverse events at 3 and 10 mg/kg we decided to continue with a lower dose of 1 mg/kg, which did not cause any signs of discomfort such as sedation or withdrawal. Mice received s.c. injections once daily of either 1 mg/kg/day AC-25793 or PBS vehicle for 4 consecutive days and a single dose of 0.3 mg/kg scopolamine or saline vehicle 30 minutes before the training session, which preceded the passive avoidance fear condition task. In scopolamine treated mice on the training day, neophobia was observed (Gutiérrez et al., 2003), which was shown by their caution to explore. To overcome this scopolamine induced neophobia, all mice were trained in the bright compartment for one minute the day before regular training, which solved all problems related to neophobia. Interestingly, treatment with AC-25793 was shown to attenuate the scopolamine induced cognitive impairment but displayed no enhanced effects on cognition, i.e. no pro-cognitive effects as defined by lack of an effect on non-scopolamine treated animals (Giessing and Thiel, 2012).

Emerging evidence suggests that reduced BDNF/TrkB signaling is associated with depression (Castrén and Kojima, 2017; Levy et al., 2018; Malhi and Mann, 2018), therefore, after the cognitive test, we performed FST to assess the antidepressant effects of AC-25793 in mice. In

the FST, a training-swimming session of 10 minutes was performed the day before the test to build up a stressor. This learned helplessness or "behavioral despair" serves as a model for depression in rodents. Animals received s.c. injections once daily of either 1 mg/kg AC-25793 or PBS vehicle for 5 consecutive days and/or a single dose of 20 mg/kg fluoxetine or saline vehicle 30 minutes before the 6 minutes FST, according to the protocol in each study. AC-25793 was shown to significantly improve the depression-like behavior (giving up swing/floating) in the FST, comparable to the effect by the antidepressant fluoxetine.

Anti-depressant like effects have been shown in the rodent FST model by several compounds. Fluoxetine (Prozac), the first approved selective serotonin reuptake inhibitor (SSRI), which blocks the reuptake of serotonin into presynaptic neurons and prolong the effects of serotonin signaling on the 5-HT receptors, demonstrates anti-depressant effects in both humans and in rodents assessed by FST (Detke and Lucki, 1996; Perez-Caballero et al., 2014) and was used as a reference compound in the FST experiments in Study III and IV.

An interesting observation regarding the studied class of steroid derivatives, is that AC-25793 and k-strophanthidin belong to the class of cardenolides with inhibitory effects on the Na^+/K^+-ATPase (the sodium-potassium pump). Indeed, all steroid derivatives with potent effects on TrkA and TrkB activation from this study (EC_{50} < 300 nM) were also potent Na^+/K^+-ATPase inhibitors. The Na^+/K^+-ATPase is important for maintaining membrane potential in cells, with physiological implications in neuronal signaling, heart rate and recently in cognition. Two separate studies and a recent patent assigned cognitive improvement effects by Na^+/K^+-ATPase inhibitors (cardiac glycosides) in humans (Chen et al., 2016; Jang et al., 2010; Jeon et al., 2017; Kaphzan, 2019; Kilander et al., 1998; Laudisio et al., 2009; Papadopoulos and Lecanu, 2012; Tohda et al., 2012).

One limitation observed in Study III is the narrow range where AC-25793 seems to have effect on cognition before adverse events appear, as demonstrated in the passive avoidance model in mice. A suggested treatment strategy would be a longer treatment period with low doses.

The findings in Study III showed that AC-25793 enhanced TrkA and TrkB signaling and it is to our knowledge the first time that increased neurotrophin signaling is shown by an Na^+/K^+-ATPase inhibitor. Future studies will explore whether the cognitive effect of these compounds is mediated via direct activation of Trk receptors, for example using a specific Na^+/K^+-ATPase inhibitor which by itself does not affect NGF/TrkA signaling.

In summary, the *in vitro* and *in vivo* testing of steroid derivatives, and especially the cardenolide AC-25793, propose a group of potent enhancers of TrkA and TrkB signaling with significant effects on cognition and depression, worthwhile to study further.

4.4 STUDY IV

The effects of ACD855 on neurotrophin signaling, cognition and depression

During assay development, screening and validation of small molecular compounds in the HTS campaign performed in Study III, we identified a promising registered drug that could enhance signaling of TrkA and TrkB. With available safety and toxicology data, this compound was selected as the lead compound for further evaluations and was denoted ACD855.

ACD855 could enhance TrkA or TrkB signaling in recombinant cells, in combination with 10 ng/ml NGF or BDNF, with an EC_{50} of 1.9 or 3.2 µM, respectively. While ACD855 could only partially increase TrkA or TrkB signaling without neurotrophin present, this suggests that the substance is rather a positive modulator of neurotrophin signaling than a pure Trk agonist.

ACD855 (20 µM) displayed significant potentiation of LTP in rat hippocampal slices after a single subthreshold theta-burst stimulation, comparable to exogenously added BDNF (50 ng/ml) as seen in figure 17. The effects by BDNF were expected since it is known that exogenously applied BDNF can induce LTP in hippocampal slices (Ikegaya et al., 2002; Ji et al., 2010), while the effects of ACD855 is a novel finding.

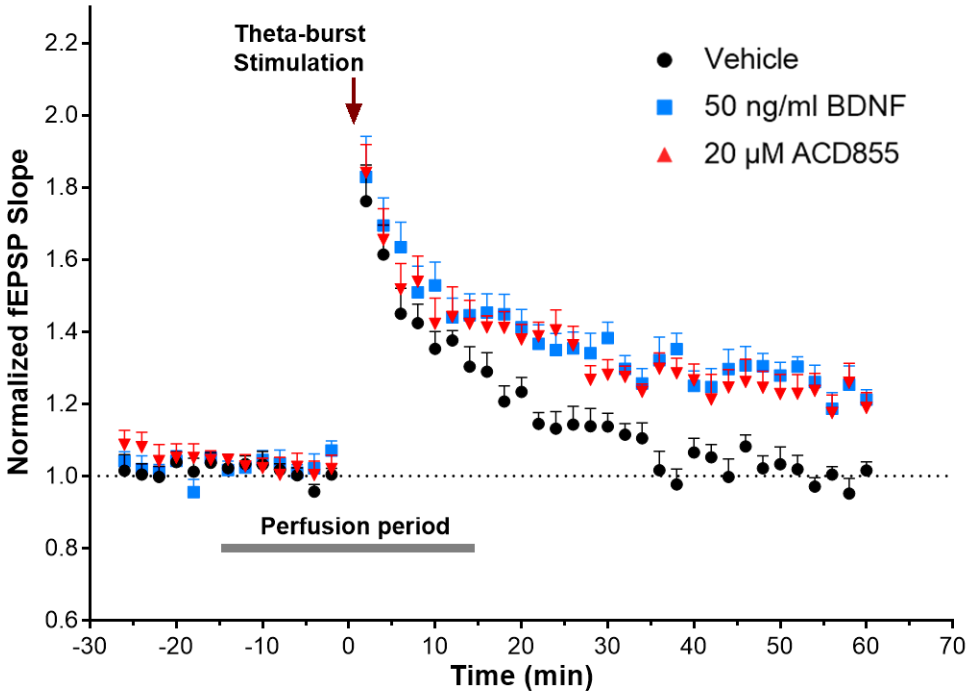

Figure 17. ACD855 elongates LTP induction in hippocampal rat slices comparable to exogenously added BDNF. Incubation with vehicle (aCSF), 50 ng/ml BDNF or 20 µM ACD855 generated an increased fEPSP slope which went back to normal levels after 35 minutes using vehicle, while both BDNF and ACD855 treatment induced an LTP (Study IV).

Microdialysis in the ventral hippocampus of Sprague Dawley rats was performed to study local *in vivo* effects of ACD855. Extracellular levels of neurotransmitters were analyzed in the microdialysis samples. Of the tested neurotransmitters (ACh, glutamate, dopamine, noradrenaline, and serotonin), ACh levels were significantly increased while the rest of the neurotransmitters were unaffected.

The enhanced effect of ACD855 on both NGF and BDNF signaling is suggested to possess the potential to restore and improve both short-term and long-term memory problems observed in AD. The short-term memory problems seen in AD is associated with the early cholinergic neuronal degeneration and reduced ACh signaling, which is suggested to be linked to disturbed NGF/TrkA signaling. While the long-term memory formation is suggested to be mediated via BDNF/TrkB signaling in the hippocampal neurons, which are also highly vulnerable to the toxic environment in the AD brain. Treatment with ACD855 aiming for increased NGF/TrkA signaling may contribute to restoration of cholinergic neurons and improved short-term memory functions, while increased BDNF/TrkB signaling may contribute to improved long-term memory processes, both at the receptor level and secondary to increased ACh signaling.

ACD855 thus potentiates NGF/TrkA and BDNF/TrkB signaling in cultured cells, induces LTP in hippocampal slices from rats and increases ACh levels *in vivo* after local microdialysis administration in the rat hippocampus.

In addition to cognitive effects by BDNF, exposure to stress and depression have shown to decrease BDNF expression in the hippocampus and the prefrontal cortex and conversely that antidepressant treatment can up-regulate BDNF in the adult brain (Duman et al., 2019).

Therefore, to further characterize ACD855 we used a panel of behavioral models to study memory functions and depression. All behavioral tests were performed after daily s.c. injections for 4-5 consecutive days with ACD855 (1, 3 or 10 mg/kg). The cognitive tests, Morris water maze and passive avoidance, were performed using young healthy C57BL/6J mice with induced cognitive impairment using scopolamine or MK-801. The depression-like test, the FST, was performed in young healthy mice or in FSL rats, which display an endogenous depression-like behavior.

In the Morris water maze, studying the swim latency parameter, ACD855 (3 mg/kg) treatment significantly attenuated the scopolamine induced cognitive impairment. Even a tendency for improved cognitive performance in cognitive normal mice was noted, shown by the significantly reduced swim latency in ACD855 treated mice day one compared with control mice. Next, the cognitive performance was studied using the passive avoidance fear conditioning test. Repeated administration of ACD855 (3 or 10 mg/kg) significantly attenuated both the scopolamine and the MK-801 induced cognitive impairment in the passive avoidance

task. Interestingly, the attenuated cognitive dysfunction by ACD855 was completely abolished when injected in combination with the TrkB antagonist, ANA-12, thus suggesting that the effect of ACD855 on cognition is TrkB dependent. Sub-effective doses of ACD855 (1 mg/kg/day) and physostigmine (single dose of 0.025 mg/kg) improved cognitive functions in the passive avoidance test, suggesting that ACD855 treatment is additive to the AChEI physostigmine. No pro-cognitive effects of treatment with ACD855 using 3 or 10 mg/kg was noted in the passive avoidance task in non-scopolamine treated animals.

Our findings that the TrkB antagonist ANA-12 eliminated the positive effects on cognitive function after ACD855 treatment in addition to the knowledge that BDNF also affects depression, made us curious to investigate whether ACD855 may have anti-depression-like effects. The depression-like effects of 3 mg/kg ACD855 (5 days treatment) was shown to be equally effective compared with a single dose of the SSRI fluoxetine (20 mg/kg) in mice, whereas in FSL rats, which have been shown to display a genetic suppression of ACh signaling (Overstreet, 1993), treatment with ACD855 displayed a significant anti-depressant effect while treatment with fluoxetine failed to reach significance. These results suggest that ACD855 can potentiate ACh signaling and counteract both cognitive problems and depression-like symptoms in rodents.

The improved effects on both cognition and depression after repeated ACD855 treatment could have synergistic effects. Cognitive decline is found accelerated in individuals with depression (Rapp et al., 2011), and individuals with depression have increased risk to develop cognitive impairments (Enache et al., 2016). Thus, depression is suggested to both contribute to AD and be secondary to the disease (Hou et al., 2010), suggesting that a BDNF signaling enhancer such as ACD855, which both improves cognition and reduces depression, could possibly display greater improvements than the expected effect of treating one condition at a time.

Previous studies have shown that NGF treatment caused low back pain and weight loss when administered intracerebroventricularly in the CNS (Eriksdotter Jonhagen, 1998) while administration directly into the brain parenchyma did not elicit pain in man (Eriksdotter-Jönhagen et al., 2012; Tuszynski et al., 2005) or rodents (Hao et al., 2000). In the periphery, muscle pain was elicited after intradermal administration to healthy volunteers (Dyck et al., 1997). The human mutations in HSAN IV (Congenital insensitivity to pain with anhidrosis, also known as CIPA) due to a null mutation in the TrkA gene (Indo et al., 1996) and the Swedish family with the HSAN V mutation in the NGF gene (Einarsdottir et al., 2004), both resulting in pain insensitivity, provided support for a link of NGF with pain mechanisms. This relation between NGF and peripheral pain has led to development of antibodies to NGF as pain therapeutics (Hefti, 2019).

Based on the above, systemic treatment with a neurotrophin signaling enhancing drug could potentially also show side effects such as pain and weight loss. When treating mice with ACD855 3-10 mg/kg/day for 4-5 days during behavioral studies, no signs of pain nor weight loss related adverse events were noted, while positive effects on cognitive dysfunction and depression-like symptoms were demonstrated. Moreover, long-term treatment of C57BL/6J young healthy mice with ACD855 3 mg/kg/day for 5 weeks did not show any weight-loss (Fig. 18) or other signs of pain or any other discomfort compared with control mice, indicating that ACD855 is suggested well tolerated in mice and also in rats.

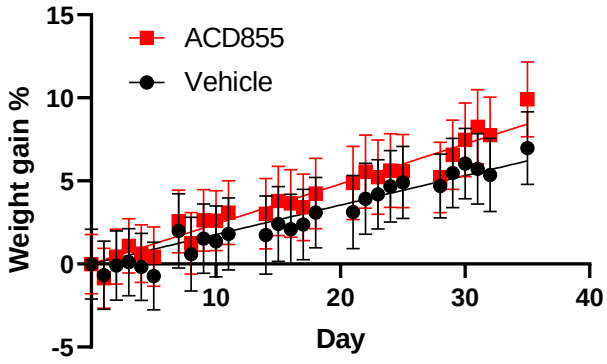

Figure 18. Injections with ACD855 3 mg/kg/day or with vehicle for 5 weeks to study weight gain/weight loss after compound treatment vs. vehicle. Data are presented as mean ± SEM (n = 16 animals per group).

Limitations: Longer treatment periods than 5 weeks was not used, but in the literature and in our own experience from NGF treatment in AD patients and in rodents, side effects of pain developed quickly during treatment and delayed responses were not seen (Eriksdotter Jonhagen, 1998).

In summary, this drug with the internal name ACD855, has been shown to display novel pharmacological effects on neurotrophin signaling, with subsequent ACh augmentation, attenuation of induced cognitive dysfunctions and anti-depressant effects.
The interesting and encouraging data on the ACD855 presented in Study IV have led to pre-clinical development of the compound, showing good safety and toxicology data after 28-days repeated dosing in rats and dogs, which provided the basis for a clinical phase 1 study in man.

5 CONCLUDING REMARKS AND FUTURE OUTLOOK

For decades, much effort has been devoted to find a disease modifying treatment for AD. During the last ten years, knowledge about the disease progress and tools to diagnose the disease have evolved, but there is still no cure available.

AD is a neurodegenerative disease characterized by Aβ plaques, neurofibrillary tangles and chronic inflammation in the brain. The main focus for drug discovery efforts has been to interfere with the amyloid pathway, which includes preventing production and aggregation, or increase the elimination of Aβ peptides. No anti-amyloid drug for the treatment of AD has yet reached the market. It is possible that AD is such a heterogeneous disease that a multi-modal approach is necessary to stop the disease progress.

The main aim of this thesis was to advance drug discovery projects within non-amyloid pathways in AD, by performing mechanistic studies, assay development and screening to identify small-molecule substances that can be used for further development of an AD treatment.

From studies in this thesis we can show that:

- A sensitive lipoxygenase assay to determine 15-LO-1 activity has been developed. DPPP was demonstrated to be a suitable substrate for detecting hydroperoxides using fluorescent analysis in HTS of small molecules targeting the 15-LO-1 protein.
- Potent 15-LO-1 inhibitors, such as BLX-000769 have been identified and may be used further to study the inflammatory response in AD.
- The NGF mutant NGF-R100E, which is a variant of the HSAN V mutant NGF-R100W, displayed more potent effects on TrkA phosphorylation in recombinant TrkA-cells, and more potent effects on proliferation, differentiation and activation of ERK1/2 in human fetal DRG neurons compared with wild-type NGF.
- Screening, validation and optimization of small-molecule compounds using the recombinant U2OS-TrkA or the U2OS-TrkB cell assays can identify compounds enhancing the NGF/TrkA or the BDNF/TrkB pathway, which translate well into effects on cognition and depression in rodents *in vivo*.
- The cardenolide AC-25793 displayed potent stimulation of both the TrkA and the TrkB receptor, enhanced neurite outgrowth in human fetal DRG neurons, attenuated cognitive dysfunction and displayed anti-depressant like effects in mice.

- AC-25793 is a Na^+/K^+-ATPase inhibitor. For the first time, it was shown that a Na^+/K^+-ATPase inhibitor increased neurotrophin signaling.
- A registered compound, named ACD855 by us, displayed novel effects on TrkA and TrkB signaling with subsequent positive effects on cognition and depression *in vivo*. ACD855 was found to be safe and well tolerated. Based on these data, ACD855 was selected for further drug development and nominated a clinical candidate. ACD855 is now in a clinical phase I study in man.

This thesis contributes to the AD field with studies on drug targets for AD.

1. Neurotrophin enhancers

Studies in this thesis have contributed with drug discovery of two potent small-molecule compounds which enhance neurotrophin signaling, demonstrating data on both *in vitro* effects and *in vivo* behavioral improvements. Amazingly, this work has led to a potential clinical drug candidate for AD. ACD855 is now in phase I development.

Both AC-25793 and ACD855 showed promising effects on cognition and depression. However, the potential of AC-25793 as an AD drug is limited due to its narrow range between an effect on cognition and adverse events, in contrast to ACD855 which displayed a much broader safety margin. The available cholinesterase inhibitors for the treatment of cognitive decline in AD are derived from the cholinergic hypothesis, in which the neurotrophin signaling plays a central role. Hence, a neurotrophin enhancer treatment has great potential to restore cholinergic neurons, enhance the neurotrophin signaling and thereby enhance the ACh signaling.

Many anti-depressants found on the market are targeting the neurotransmitters serotonin, norepinephrine and dopamine, including the SSRIs. The effect of SSRIs is often delayed and it usually takes several weeks before the treatment effect is achieved, while the anesthetic compound ketamine displays rapid anti-depressant effects at low doses via inhibition of the NMDA receptor (Murrough, 2012). Unlike other anti-depressants, the effects of ketamine last significantly longer than the half-life of the compound. Emerging evidence suggest that the inhibition of excessive glutamate signaling via NDMA receptors by ketamine reduce the Ca^{2+} influx and increase BDNF expression, shown in mice, rats and humans, and may reflect BDNF triggered synaptic plasticity effects (Autry et al., 2012; Björkholm and Monteggia, 2016; Garcia et al., 2008; Woelfer et al., 2019). The anti-depressant effect of our identified neurotrophin signaling enhancers, AC-25793 and ACD855, may induce BDNF derived

synaptic changes and anti-depressant effects without side effects related to ketamine or SSRI treatment.

2. "Painless" NGF mutant

Another approach worth further investigating as a potential therapy target for AD is the NGF mutant NGF-R100E, with its stimulatory effects on TrkA and its neurite outgrowth abilities, in addition to not affecting pain signaling.

3. 15-LO-1 inhibitors

The third contribution was the identification of 15-LO-1 inhibitors as potential future drug targets aiming at affecting inflammation in AD. The first step to identify 15-LO-1 inhibitors was to develop a sensitive lipoxygenase assay for HTS screening for 15-LO-1 activity, which has been achieved. Several 15-LO-1 inhibitors were tested as part of the development of the assay. One compound, BLX769, has been used by other researchers to detect 15-LO-1 activity.

Targeting the inflammation in AD is a struggle with both beneficial and harmful events. The 15-LO-1 target is a puzzle with suggested pro- and anti-inflammatory arachidonic acid metabolites. At the same time, a target cannot be fully investigated until the hypothesis is tested. Now, there is a useful and sensitive assay available for determining 15-LO-1 activity which allows further development of the contribution of the 15-LO inhibitors to inflammatory treatment in AD.

In conclusion, non-amyloid targets are highly interesting treatment strategies for AD. Here, enhancers of both TrkA and TrkB signaling are shown to be particularly promising. Non-amyloid treatments for AD might also be an adequate complement also if, or when, an amyloid-treatment is available. Since the disease is complex, multi-factorial and the timing of various treatment might be of great importance, a combination therapy targeting different mechanisms of AD may be the answer.

To cure AD is the ultimate dream, but I believe that everything we can do to improve the quality of life for the AD patients is well worth the effort.

6 ACKNOWLEDGEMENTS

This doctoral thesis has been conducted at the Division of Clinical Geriatrics, Department of Neurobiology, Care Sciences and Society, Karolinska Institutet in collaboration with AlzeCure Foundation.

I would like to take the opportunity to express my sincere gratitude to everyone who contributed to the completion of my doctoral thesis.

Professor **Maria Eriksdotter**, my main supervisor. First of all, I would like to thank you for accepting me as a PhD student and for your encouragement and support during these years. Thank you for sharing outstanding knowledge in NGF treatment, the Swedish Dementia Registry (SweDem) and scientific writing. Thanks for all invitations to your home and the fruitful Mia meetings.

Dr. **Pontus Forsell**, my main co-supervisor. You have taught me more or less everything I know about preclinical drug discovery. Your positive attitude is exceptional, thank you for cheering up every day at work. With clever ideas and hard work, you create everything from a pool in your garden to eminent drug discovery projects. You are a true role model and a fantastic coach.

Professor **Angel Cedazo-Minguez**, my co-supervisor. You are a visionary in research and drug discovery, I wish we had collaborated more. I always get inspired when we meet and talk.

My mentor **Gunilla Ekström**. Thank you for drug discovery inspiration all the way from Astra Pain Control via Biolipox to the defense of my thesis.

Special thanks to AlzeCure Foundation, AlzeCure Discovery AB, AlzeCure Pharma AB, Sinfonia Biotherapeutics AB, the inventors and great friends thereof. Pontus Forsell again, **Gunnar Nordvall**, **Johan Lundkvist**, **Johan Sandin** and **Magnus Halldin**, AlzeCure is my second home by now and I love your company. **Nather Madjid**, you are a great tutor and a one-of-a-kind *in vivo* guru. Mice and rats were not my friends before I met you, but now I can collaborate with them, look them in their eyes and say that we are a team by now. **Sven Ove Ögren**, thank you for sharing so much knowledge in behavioral science. The AlzeCure crowd is now extended and include these fantastic girls, **Petra Verhagen**, **Cristina Parrado**

Fernandez, **Maria Backlund** and **Veronica Lidell**. Last, but not least, thank you **Martin Jönsson** CEO at AlzeCure Pharma AB, who will guide us all to perform our best.

Professor **Bengt Winblad** and Swedish Brain Power for supporting a PhD position at AlzeCure. Dementia and Alzheimer's disease would be even worse without you.

I like to thank all my present and previous co-workers at the Division of Clinical Geriatrics, especially **Eric Westman**, **Anette Eidehall**, **Daniel Ferreira Padilla**, **Olga Voevodskaya** and **Azadeh Karami**. And my co-authors and co-workers at the Division of Neurogeriatrics, especially **Erik Sundström**, **Elisabet Åkesson**, **Maria Ankarcrona**, **Erika Vazquez-Juarez**, **Mia Lindskog**, **Henrik Biverstål**, **Erik Hjorth**, **Marianne Schultzberg**, **Ceren Emre**, **Johanna Wanngren**, **Jolanta Lundgren**, **Chenhong Lin**, **Eva-Britt Samuelsson**, **Lena Holmberg**, **Anna Matton** and **Helena Karlström**.

I like to thank former colleagues, co-authors and close friends, **Daniel Forsström**, **Malin Johannesson**, **Yasmin Huque-Andersson**, **Marie Björk**, **Erik Silfverplatz**, **Andrei Sanin**, **Wesley Schaal**, and **Benjamin Pelcman**.

Hans-Erik Claesson, for introducing me to academia research and the world of arachidonic acid metabolites.

I would like to thank all my friends from near and far. I know I will miss at least a few if I try to mention some of you. From the deepest of my heart, thank you for all the good times and the bad times, for all the fun we have had and will have in the future. I hope to interact much more from now on!

My American cousins, especially **Elizabeth**. Thank you for being an extra mother to my kids, your warm heart sends blessings across the Atlantic all year around. My cousins and beloved families, **Börje**, **Eva**, **Jonas**, **Linnea**, **Hugo**, **Oscar**, **Wilmer**, **Mats**, **Maria**, **Emelie**, **Meja**, **Elise**, **Angelica**, **Göran**, **Saga**, **Tomas**, **Otto** and **Julia**. My Swedish-Japanese little brother, **Yuki**, I am so glad you are back in Sweden. You are all part of my closest family and you are always on my mind. My home will be open for another fika as long as I live.

My mother **Mari**, you are amazing is so many ways. Thank you for taking so well care of me and my family, your support is endless. Since early in my life you have acted as both my mother and father and you have lovingly guided me through life.

Anna, my sister and best friend. **Ingrid** and **Gunnar**, all the best to you!

The past year has been a challenge for my family and I dearly appreciate your encouragement. **Mattias**, for your love.

Thank you for the music, the songs I'm singing
Thanks for all the joy they're bringing
Who can live without it? I ask in all honesty
What would life be?
Without a song or a dance, what are we?
So, I say thank you for the music, for giving it to me

Eternal love to my amazing children.
August, my one-and-only son. Your dedication to succeed is astonishing. Veni, vidi, vici!
Elvira, my hero. Your warm heart will guide you through life. You can and you will, always!
Frida, my baby. Your energy cheers up every day of my life. Let’s prepare for next party!

This work was supported by funds from Alzheimer’s Drug Discovery Foundation, Vinnova (2014-00347), Swedish Brain Power, the Swedish Brain Foundation (Hjärnfonden), the Swedish research council (grant # 2016-02317), and the Regional Agreement on Medical Training and Clinical Research (ALF) between Stockholm County Council and Karolinska Institutet.

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